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IMPACT OF ANAEROBIC CONDITIONS AND MICROBIAL ACTIVITY ON THE BIOAVAILABILITY OF HIGH MOLECULAR WEIGHT POLYCYCLIC AROMATIC HYDROCARBONS IN SOILS

by

Tasha Lara Pravecek

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Sciences and Engineering, School of Public Health

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2000

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ABSTRACT

Tasha Lara Pravecek: Impact of Anaerobic Conditions and Microbial Activity on the Bioavailability of High Molecular Weight Polycyclic Aromatic Hydrocarbons in Soils
(Under the direction of Frederic K. Pfaender)

The influence of imposed anaerobic conditions on aqueous phase polycyclic aromatic hydrocarbon (PAH) fate was investigated. Highly aged, PAH contaminated soil was incubated with an oxygen scavenging titanium(III) citrate complex, or water, or water that contained nitrate or sulfate in combination with a H_2(g), N_2(g), or N_2:CO_2(g) (80:20) headspace. The anaerobic conditions imposed in this study resulted in increased aqueous concentrations of fluoranthene, pyrene, benz[*a*]anthracene and benzo[*a*]pyrene. Benz[*a*]anthracene and benzo[*a*]pyrene were above aqueous solubility limits, by as much as an order of magnitude for the latter. The mechanism for this increased PAH solubility is hypothesized to be a combination of oxidation-reduction potential and microbially mediated pH alteration resulting in enhanced solubility of organic material with associated PAH. Methanogenic organisms and sulfate reducing bacteria were seen to have the most significant effect on an increase in aqueous phase PAH. In addition, incubations that changed from anaerobic to aerobic conditions assessed the effect of anaerobiosis conditions on bioavailability. The solvating effect of formalin, used as a metabolic inhibitor, was also examined.

The bioavailability of pyrene in three pristine soils was examined under aerobic and anaerobic conditions. Three soils were aged with pyrene and [^{14}C]pyrene for 65 days, then incubated with water, nitrate, or sulfate under aerobic or anaerobic conditions for one year. Under aerobic conditions, microorganisms in two soils mineralized 58-82% of added [^{14}C]pyrene. Nitrate treatment was seen to enhance the initial mineralization rates in these two soils. In one soil, the non-extractable pyrene decreased over the course of the study due to desorption and mineralization. Nitrate amendment was shown to enhance the rate of mineralization from the non-extractable fraction in this soil. Under anaerobic conditions

generated with electron acceptors and N_2:CO_2(g) headspace, two soils showed a treatment related increase at 365 days in the extractable [^{14}C]pyrene, presumably due to microbially mediated ORP and pH alteration of the soil.

Anaerobic conditions and anaerobic microbial communities were shown to enhance the aqueous solubility of PAH in soil and extractability of pyrene from soil, which may influence bioavailability and transport of PAH in the environment.

To my parents, Evelyn Joan and Lawrence Ernest Pravecek,
this dissertation is dedicated with love…
you both gave me skills to achieve success.

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LIST OF ABBREVIATIONS

Air/An	Aerobic switched to Anaerobic headspace
An/Air	Anaerobic switched to Aerobic headspace
BES	Bromoethanesulfonic acid
DCM	Dichloromethane
DOM	Dissolved Organic Material
ED	Soil from Edwards Air Force Base, CA
E_H	Measure of Oxidation-Reduction Potential
HOC	Hydrophobic Organic Compounds
HPLC	High Performance Liquid Chromatography
NAPLs	Non-Aqueous Phase Liquids
MN Soil	Soil from Reilly Tar and Chemical Corporation Superfund Site, MN
ORP	Oxidation-Reduction Potential
PAH	Polycyclic Aromatic Hydrocarbons
SD	Soil from Winner, SD
SK	Soil from Schenck Forest, Raleigh, NC
SRB	Sulfate-Reducing Bacteria
Ti-citrate	Titanium(III) citrate oxygen scavenging complex
TOC	Total Organic Carbon

CHAPTER 1: INTRODUCTION AND OBJECTIVES

1.1. INTRODUCTION

The environmental and human health significance of polycyclic aromatic hydrocarbon (PAH) contamination of soils and sediments is well established [5, 6, 10, 15, 46, 88]. In general, the persistence of PAH is increased in soil or sedimentary environments containing natural organic matter [12, 18, 33, 45]. As microorganisms capable of degrading PAH are abundant in the environment, association of PAH via sorption, partitioning or entrapment with soil or sedimentary organic matter is believed to be responsible for decreased microbial availability [15, 46, 88]. The formation of bound PAH-residues has been reported in the literature [5, 6, 10, 12, 19, 33, 45]. Some researchers suggest that microorganisms facilitate the sequestration of xenobiotics in an attempt to reduce the toxicity of a contaminant or its metabolic products [12, 20].

Any condition that enhances metabolic activity, decreases soil binding of PAH, or renders bound-PAH more accessible to microorganisms would theoretically increase the potential for biodegradation. PAH that are sequestered or otherwise non-bioavailable may be degraded in soils with specific organic matter characteristics. For instance, Manilal and Alexander report phenanthrene mineralization when it is sorbed to muck soil, which indicates that PAH is degraded even in a sorbed state [56]. Addition of inorganic nutrients, such as nitrate or sulfate, may also stimulate microbial growth and enhance biodegradation of organic compounds under aerobic or anaerobic condition [56]. Anaerobic conditions may enhance bioavailability of PAH through microbial mediation or oxidation-reduction influence on soil systems. Anaerobiosis is a condition whose effect on the microbial availability of soil-bound PAH are not adequately addressed in the literature.

Soils and sedimentary organic material may experience anaerobic conditions depending upon depth in the soil/sedimentary column and, in the case of soil, under flooded hydrological conditions. The thermodynamic sequence of oxidation-reduction reactions

mediated by microbes is well established [55, 75]. The microbial community in a soil environment may alter soil structure [9] and make bound PAH more accessible in the soil phase, or the aqueous phase either as dissolved PAH or as PAH sorbed to "solubilized" organic matter. In addition, anaerobic metabolism that employs H_2(aq) as electron acceptor may cause local pH increases leading to enhanced dissolution of soil organic matter. For example, Burgess and others [16] found that PCB-colloid interactions were more pronounced with increased core depth. These workers and others [16, 30, 38, 61] have observed higher than expected PAH concentrations in an aqueous phase containing soluble natural organic matter, which suggests the existence of PAH-organic soluble complexes. Some authors have shown a decrease in bioavailability to aquatic bluegills, guppies and amphipods due to PAH binding with dissolved organic material (DOM) [29, 48, 57]. However, no reports exist that show a decreased bioavailability of DOM-associated PAH to microorganisms under either aerobic or anaerobic conditions.

1.2. OBJECTIVES

The first objective of this work was to examine the influence of controlled anaerobic conditions on the aqueous solubility of high molecular weight PAH during a one-year incubation of contaminated soil. My approach was to generate controlled anaerobic conditions using (a) anaerobic headspace or, (b) anaerobic headspace and an oxygen-scavenging complex (titanium(III) citrate) in the aqueous phase or, (c) anaerobic headspace and electron acceptor amendments in the aqueous phase. These three methods to generate anaerobic conditions may cause different PAH solubility results due to microbial community structure or method-induced abiotic effects. The anaerobic headspaces used were N_2:CO_2(g) (80:20), N_2(g), or H_2(g). I selected these gases because of their availability and possible differences concerning oxidation-reduction potential (ORP) generation and potential microorganism induction. Oxygen scavenging complexes are chemical substances that obtain a relatively precise ORP. And, electron acceptor amendments induce the most thermodynamically favored consortia of microorganisms to dominate a system. Each treatment profile was compared to an equivalent control with metabolic inhibitors. The second objective of this work was to assess aerobic bioavailability of PAH made more soluble under anaerobic conditions. The experimental protocol of the first two objectives

evaluated the effect of anaerobic conditions and microbial activity on PAH solubility, and is addressed in Chapter 3.

The objectives of the second set of studies were to examine the influence of (a) aerobic conditions and inorganic nutrient addition, and (b) anaerobic conditions and electron acceptor amendment, on the bioavailability of pyrene in three distinctly different soils during a one-year incubation. Three previously uncontaminated soils were aged with pyrene and [^{14}C]pyrene for 65 days, then incubated with water, NO_3^-, or SO_4^{2-} under aerobic or anaerobic conditions for one year. Pyrene was selected as a representative high molecular weight PAH. Anaerobic conditions were generated by N_2:CO_2(g) headspace and water, or NO_3^- or SO_4^{2-} amended aqueous phase. Each treatment profile was compared to an equivalent control with metabolic inhibitors. The experimental protocol evaluated (a) the effect of inorganic nutrient addition on pyrene bioavailability and biodegradation, and (b) the effect of anaerobic conditions and anaerobic microbial communities on pyrene associations with soil, and is addressed in Chapter 4.

Chapter 5 provides the summary and conclusions of this research project.

CHAPTER 2: LITERATURE REVIEW

2.1. POLYCYCLIC AROMATIC HYDROCARBONS (PAH)

2.1.1. STRUCTURES AND PHYSICAL PROPERTIES

PAH are organic chemical compounds composed of two or more fused benzene rings. They are thermodynamically stable due to their large resonance energy. The aromaticity of PAH also makes them fluorescent and easily photo-oxidized [64]. However, since PAH are non-polar, hydrophobic compounds, they have low water solubility and therefore associate primarily with particle surfaces in the environment. This association makes PAH less likely to be affected by volatilization, photolysis, and biodegradation, thus contributing to their persistence in the environment [21, 64]. Some physical properties of PAH are listed in Table 2.1, on the following page.

High molecular weight PAH are those with four or more rings, or molecular weight of 202 or greater. Figure 2.1 presents the high molecular weight PAH addressed in this dissertation.

2.1.2. ORIGINS

Due to their widespread use and generation, PAH are ubiquitous environmental contaminants. Natural sources of PAH include oil seeps, forest and prairie fires, and some plant and bacterial reactions [21, 91]. Anthropogenic origins of PAH include petroleum products and storage, coal tar and coal processing wastes, manufactured gas plants (MGP), fossil fuel combustion, tobacco and cigarette smoke, smoked food and roasted coffee, creosote and wood preservative wastes, and other fossil fuel related sources [1, 2, 21, 25, 64, 85, 91].

Table 2.1. Physical Properties of Some High Molecular Weight PAH.

Compound	M.W. ($g\text{-}mole^{-1}$)	Vapor Pressure (Pa)	Log K_{ow}	Aqueous Solubility ($mg\text{-}L^{-1}$)
Fluoranthene	202.3	8.7x10-3	5.22	0.26
Pyrene	202.3	0.012	5.18	0.13
Benz[a]anthracene	228.3	6.0x10-4	5.91	0.011
Benzo[a]pyrene	252.3	2.1x10-5	6.04	0.0038

Adapted from [53].

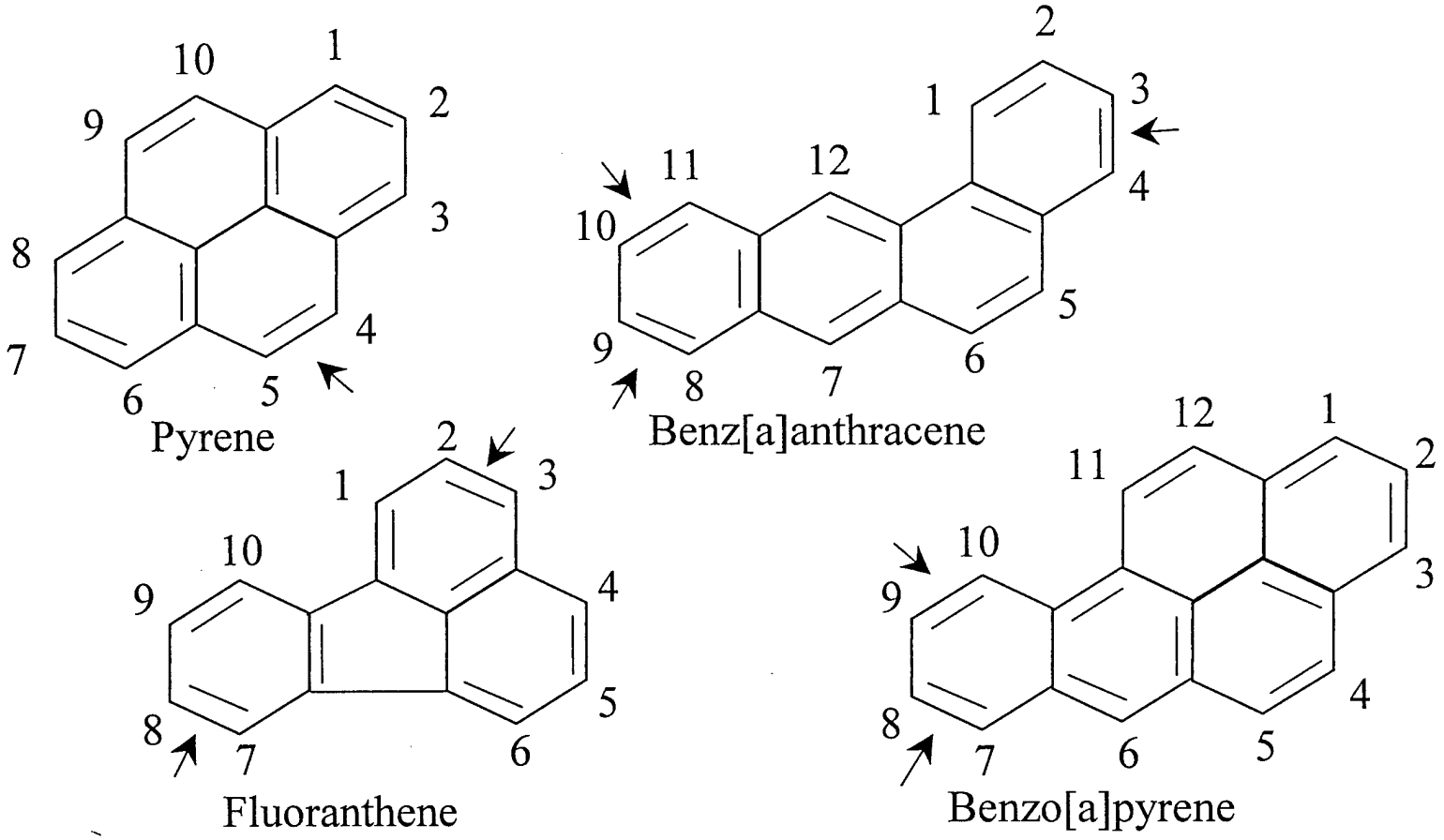

Figure 2.1. Structures of some high molecular weight polycyclic aromatic hydrocarbons. Arrows indicate primary sites of metabolic attack [79].

2.1.2. TOXICITY

PAH pose significant environmental and health problems. In 1775, Percival Pott identified the link between chimney sweeps' exposure to soot and "industrial disease" or cancer [7, 25]. Later, in 1875 von Volkman proved a link between German workers exposed to coal tar and skin cancer [7]. It is now well known that many PAH are mutagenic or carcinogenic and thus present a significant human health hazard [8]. For example, benzo[a]pyrene has been extensively studied with regard to its metabolism and subsequent carcinogenicity. Benzo[a]pyrene is metabolized by many isozymes of cytochrome P450, producing at least 15 major phase I metabolites [35]. One of the carcinogenic metabolites of benzo[a]pyrene is produced when benzo[a]pyrene is metabolized by P450 to benzo[a]pyrene-7,8 epoxide, then hydrated by epoxide hydrolase to form benzo[a]pyrene-7,8-diol. The benzo[a]pyrene-7,8-diol is metabolized by P450 to form benzo[a]pyrene-7,8-diol-9,10-epoxide which binds preferentially to deoxyguanine residues of DNA. The epoxide is known to be carcinogenic in rodents and mutagenic in eukaryotic and prokaryotic cells [8, 35].

Some PAH have been listed by the U.S. Environmental Protection Agency as priority pollutants to be monitored in industrial effluents [19, 21, 59, 91]. The priority pollutant PAH considered in this dissertation include: fluoranthene, pyrene, benz[a]anthracene, and benzo[a]pyrene. Indicative of their ubiquitous nature, the following were detected in 2617 industrial wastewater samples analyzed (percent in parenthesis): fluoranthene (7.2), pyrene (7.8), benz[a]anthracene (2.3), and benzo[a]pyrene (3.2) [25].

2.1.4. PERSISTENCE IN SOIL

In general, the persistence of PAH is increased in soil or sedimentary environments that contain natural organic matter [5, 50, 62, 87]. Since PAH are hydrophobic compounds, they associate with sediments or soils [21]. The exact mechanisms of this association are unknown, however many theories exist to explain the phenomenon. Some theories include sorption or partitioning into solids in the environment, partitioning into nonaqueous-phase liquids (NAPLs), or entrapment within the physical matrix of the soil in the form of bound residues or simple diffusion into a micropore [6, 33, 51]. The behavior of the PAH in the

soil will differ based on the soil properties, including organic matter and mineral matrix present.

The characteristics of the soil organic matter (SOM) will determine the sorption and partitioning behavior of the PAH. SOM is a relatively homogeneous, highly amorphous, lipophilic, gel-like matrix [51]. The polarity and aromatic content of SOM varies, and may control the reactivity with hydrophobic organic compounds. For instance, soil organic matter with oxygen-containing functional groups will alter reactivity, partitioning, and sites for covalent bonding [86]. The affinity of SOM for hydrophobic organic compounds, such as PAH, depends on the origin and diagenetic history of the soil organic matter [47].

Sorption coefficients for some compounds are often higher than predicted for the amount of soil organic matter present, which suggests that the mineral phase contributes significantly to sorption [54]. Nonionic hydrophobic organic compounds (HOC), such as PAH, are not believed to interact directly with mineral surfaces, but instead with attached natural organic matter (NOM) or with the vicinal water. Holmen and Gschwend [37] suggest that organic matter-rich iron oxyhydroxide and aluminosilicate clay coatings are the principal HOC sorption media in aquifer sands. In addition, Bollag and Loll [12] report that in soils with low organic matter content, clays compete with humics for binding contaminants. Determining whether PAH is associated with SOM or mineral phases in soil is important in site assessment and development of remediation strategies.

2.1.5. TRANSPORT AND FATE

"The possible fates of PAHs in the environment include volatilization, photooxidation, chemical oxidation, bioaccumulation, adsorption to soil particles, leaching, and microbial degradation," [21]. The PAH associated with soil may dissolve or partition into the aqueous phase (pore water) based on the aqueous solubility and octanol-water partition coefficient (K_{ow}), presented in Table 2.1. Since the aqueous solubilities of high molecular weight PAH are very low, only minimal aqueous phase concentrations of PAH are predicted. However, many researchers report higher than expected concentrations in the aqueous phase in the presence of DOM, which suggests possible sorption of PAH to the DOM [16, 30, 38, 61]. The binding affinity of an organic contaminant to particles in the aqueous phase is related to its K_{ow} and the organic content of the particles [57, 61].

Gauthier, et al. [30] further defined this interaction by showing that PAH became bound more strongly to humic materials that possess a high degree of aromaticity. In any case, colloids and other dissolved organic matter are important because of their high specific surface area, which give them a high potential to adsorb hydrophobic organic compounds such as PAH [16, 61].

2.1.6. MICROBIAL BIOAVAILABILITY

As microorganisms capable of degrading PAH are abundant in the environment, association of PAH via entrapment, sorption, or partitioning in soil or sedimentary organic matter is believed to be responsible for decreased microbial availability [15, 46, 88]. PAH can diffuse into the nanopores of soil particles. Soil scientists define nanopores as any pore smaller than micropores that have diameters of 5-30μm. Bacteria and fungi have diameters greater than 1μm. If a compound diffuses into the smaller diameter nanopores, no microorganisms will be present to degrade them. As the PAH diffuses out of a nanopore, it may follow a very lengthy and tortuous path, frequently sorbing and desorbing from the pore wall [5]. The fraction of HOC in these nanopores is unknown, and may be a significant quantity. Therefore, degradation is limited by the time it takes for a PAH to diffuse out of a nanopore to a location where microorganisms are present.

The formation of bound PAH-residues, non-extractable PAH fractions, has been reported in the literature [5, 6, 10, 12, 19, 33, 45]. Some researchers suggest that microorganisms facilitate the sequestration of xenobiotics in an attempt to reduce the toxicity of a contaminant or its metabolic products [12, 20]. It is thought that oxygen-containing functional groups incorporated into the parent PAH through microbial degradation may facilitate the sequestration process [72, 73]. Many authors report that SOM-associated PAH are not available to pose a toxic threat or be degraded by microorganisms [22, 33, 34, 46, 80, 81].

PAH interaction with dissolved organic matter may influence bioavailability to aquatic organisms [29, 48, 57]. McCarthy and Jimenez [57] discovered a decreased bioaccumulation of naphthalene and benzo[a]pyrene by bluegill sunfish in the presence of 20mg C/L dissolved humic material. They postulated that the PAH bound to the dissolved particles was less available for uptake by organisms. Their results also show that the

influence of dissolved humic material is greater for benzo[a]pyrene than naphthalene [57]. So, one would expect the influence of dissolved organic matter to be more important with higher molecular weight PAH. Landrum [48] reported reduction in bioavailability of PAH to the amphipod *Pontoporeia hoyi* in the presence of dissolved organic matter. He postulated that the dissolved organic matter-PAH complex was either too large or too polar to flow past the respiratory membrane or integument of the organism. No studies have been reported that show a decreased bioavailability of dissolved organic matter associated PAH to microorganisms.

2.1.7. AEROBIC PAH DEGRADATION

The major decontamination route of PAH in soil and sediment involves aerobic microbial degradation [21]. PAH are aerobically transformed by microorganisms to dead-end metabolites or mineralized to carbon dioxide. For example, pyrene is mineralized by *Mycobacterium* sp. strain PYR-1 through a series of metabolic reactions that begin with the oxidation of the 4 and 5 positions by a dioxygenase, which forms a cis-dihydrodiol, or catechol-like structure [21, 79]. This is followed by multiple ring cleavages to metabolically utilizable substrates and CO_2(g) [74]. Oxidation at the 1 and 2 positions leads to a dead end metabolite, 4-hydroxyperinapthenone [21, 79]. The initial sites for attack and diol formation are indicated for other high molecular weight PAH in Figure 2.1 [79]. High molecular weight PAH are more recalcitrant than other PAH because they desorb more slowly based on K_{ow} and aqueous solubility.

The addition of inorganic nutrients can sometimes stimulate microbial proliferation and PAH metabolism [5, 56, 64]. PAH-contaminated sites are often nutrient limited. Manilal and Alexander [56] found that phenanthrene mineralization was enhanced with phosphate addition. Inorganic nitrogen addition to enhance aerobic degradation is often reported for *in situ* bioremediation efforts [5, 64].

2.1.8. ANAEROBIC PAH DEGRADATION

Anaerobic degradation has previously been reported only in low molecular weight PAH, and fluoranthene, and no investigators have reported anaerobic degradation of any other high molecular weight PAH [91]. Many authors report degradation of low molecular weight PAH under nitrate-reducing, sulfate-reducing, or methanogenic conditions [3, 23, 24, 31, 49, 59, 60]. Table 2.2 presents a compilation of anaerobic degradation reported for PAH.

Table 2.2. Anaerobic Degradation of PAH

Compound	Oxidation-Reduction Condition	Soil Type	Endpoint	Headspace Gas	Reference
Naphthalene	Nitrate-reducing	Oil-cont. soil	$[^{14}C]$-CO_2	N_2	[3]
	Nitrate-reducing	Grassland	Disappearance	He	[59, 60]
	Nitrate-reducing	Sediment-column	Disappearance	N/A	[49]
	Sulfate-reducing	Marine sediment	$[^{14}C]$-CO_2	N_2CO_2 (95:5)	[24]
	Sulfate-reducing	Marine sediment	$[^{14}C]$-CO_2	N_2CO_2 (95:5)	[24]
	Sulfate-reducing	Marine sediment	$[^{14}C]$-CO_2	N_2CO_2 (95:5)	[23]
	Sulfate-reducing	Sediment-column	Disappearance and $[^{14}C]$-CO_2	N/A	[49]
	Manganese-reducing	Sediment-column	Disappearance	N/A	[49]
	Methanogenic	Enrichment culture	Disappearance	Not stated	[31]
Acenaphthalene	Nitrate-reducing	Grassland	Disappearance	He	[59, 60]
Phenanthrene	Sulfate-reducing	Marine sediment	$[^{14}C]$-CO_2	N_2CO_2 (95:5)	[23, 24]
Methyl-naphthalene	Sulfate-reducing	Marine sediment	$[^{14}C]$-CO_2	N_2CO_2 (95:5)	[24]
Fluorene	Sulfate-reducing	Marine sediment	$[^{14}C]$-CO_2	N_2CO_2 (95:5)	[24]
Fluoranthene	Sulfate-reducing	Marine sediment	$[^{14}C]$-CO_2	N_2CO_2 95:5	[24]
			PAH Not Degraded		
4-5 ring PAH	Nitrate-reducing Sulfate-reducing Methanogenic	Enrichment culture	Disappearance	Not stated	[31]
Pyrene	Sulfate-reducing	Marine sediment	$[^{14}C]$-CO_2	N_2CO_2 (95:5)	[24]
Benzo[a]pyrene	Sulfate-reducing	Marine sediment	$[^{14}C]$-CO_2	N_2CO_2 (95:5)	[24]

2.2. ANAEROBIOSIS: EXPLANATION AND GENERATION

2.2.1. OXIDATION-REDUCTION POTENTIAL MEASUREMENT AND E_H

The oxidation-reduction potential, ORP or E_H, of a soil system or culture is a complex interaction between the acids, bases, and buffers within that system [32]. The E_H is measured with an ORP probe, which consists of a platinum electrode and a reference electrode (ex. Ag/AgCl(s)), attached to a potentiometer, such as a pH meter. E_H is an intensity gauge measuring the availability rather than the quantity of electrons [11]. When analyzing a soil system, the ORP probe measures a poorly defined average of all the oxidation-reduction (redox) couples present. Often the redox data are presented as a "mixed potential" [11].

The redox potential is pH-sensitive, therefore both the measured redox potential and pH should be reported [11]. Jacob relates that a one-unit pH increase causes the redox potential to become more negative by 57.7mV [41]. To explain, reactions that convert oxidized species to more reduced species consume hydrogen ions, which results in increased pH. The change in pH with more reduced conditions may have important implications to the microorganisms and also the structure of soil in the immediate area.

2.2.2. NATURALLY OCCURRING ANAEROBIC CONDITIONS

Soils and sedimentary organic material may experience anaerobic conditions depending upon depth in the soil/sedimentary column and, in the case of soil, under flooded hydrological conditions. Anaerobic microsites also exist within the soil particles. Kaspar and Tiedje [44] describe two main causes of soil anaerobiosis: (1) a high rate of oxygen consumption caused by a high respiration rate that generally correlates with a relatively high organic matter content, and (2) a low rate of gas diffusion. The presence of water in the soil facilitates anerobiosis by effecting both conditions.

The thermodynamic sequence of redox reactions mediated by microbes is well established [11, 55, 75]. As microorganisms deplete electron acceptors the next most energetically favorable group of organisms dominates. Oxygen is the most thermodynamically efficient electron acceptor. After oxygen is depleted by aerobic respiration, denitrification begins when the redox falls to +747 mV. Denitrifying bacteria

use nitrate as an alternative electron acceptor and when nitrate is depleted, reduction of Mn^{4+} begins below a redox of +526 mV. Mn^{4+} depletion is followed by reduction of Fe^{3+} at -47 mV [75]. Iron reduction is found in fresh, brackish, and marine habitats. A wide variety of microorganisms, which include fungi, facultative anaerobic bacteria, and strict anaerobes are capable of reducing iron. Next on the energy ladder are the sulfate-reducing bacteria. The sulfate-reducing bacteria (SRB) couple organic matter oxidation with reduction of sulfate. These bacteria are ubiquitous in the environment, however the majority of isolates have been obtained from marine habitats where sulfate is rarely limiting [78]. Finally, after depletion of all other electron acceptors, the methanogens and acetogens use carbon dioxide as their electron acceptor. Methanogenesis is the process by which carbon dioxide is reduced to methane [78].

2.2.3. LABORATORY INDUCED ANAEROBIC CONDITIONS

In the laboratory, anaerobic conditions can be artificially induced and controlled. If an aerobic system is sealed, oxygen is eventually depleted by aerobic organisms. Alternatively, one of the following approaches can be used to generate controlled anaerobic conditions: (a) anaerobic headspace or, (b) anaerobic headspace and an aqueous phase oxygen-scavenging complex (ex. titanium(III) citrate) or, (c) anaerobic headspace and aqueous phase electron acceptor amendments.

There is no "industry standard" for anaerobic headspace gases for microcosms. Most anaerobic chambers use a $H_2(g):N_2$ (g) (4%:96%) since hydrogen is used with a palladium catalyst to scavenge oxygen. The level of 4% $H_2(g)$ is selected for safety reasons because this is below the flammability limit of hydrogen. Many laboratories use $N_2:CO_2(g)$ (80%:20%) or $N_2:CO_2(g)$ (95%:5%) as the headspace for microcosms because these mixtures are inexpensive and readily available. In many cell culture studies, $N_2:CO_2(g)$ (80%:20%) is used to maintain anaerobic conditions and offer a buffering capacity from the carbon dioxide. In the studies presented in this dissertation, $N_2:CO_2(g)$ (80%:20%), $N_2(g)$, and $H_2(g)$ were selected.

Oxygen scavenging complexes are chemical substances that may obtain a relatively reproducible redox potential. Titanium(III) citrate has been used as an oxygen scavenging complex and redox indicator because it is a strong reducing agent (E_H = -480 mV) and is

relatively non-toxic to selected anaerobic microorganisms at high concentrations [42, 44, 92, 94]. Cysteine-dithiothreitol has also been used as a redox buffer because it also generates a reduced environment (E_H = -210 to -330 mV). The cysteine-dithiothreitol medium has been reported to be successfully used in anaerobic incubations [90]. There are several other oxygen-scavenging complexes, however few are non-toxic to microorganisms. Despite the reported E_H values of these couples, the redox of a system or microcosm is also controlled by the soil component redox couples, aqueous phase additions (eg. mineral medium), and microbially mediated redox reactions.

Under anaerobic conditions, a terminal electron acceptor amendment may induce the most thermodynamically favored consortia of microorganisms to dominate a system [55], as described in section 2.2.2. These microorganisms mediate a specific oxidation-reduction potential [63]. Table 2.3 presents free energy changes for some redox reactions mediated by microorganisms.

Table 2.3. Free Energy Changes for Reactions Mediated by Microorganisms.

Reaction Mediated	$\Delta G^{\circ\prime}$ (kJ-mole^{-1})
Nitrate-Reducing Bacteria	
$NO_3^- + 2\{CH_2O\} + 2H^+ \rightarrow NH^{4+} + 2CO_2 + H_2O$	-655.0
Acetogenic Bacteria	
$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + 3H_2 + HCO_3^-$	76.1
$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$	-54.95[a]
$4H_2 + H^+ + 2HCO_3^- \rightarrow CH_3COO^- + H_2O$	-104.6
Methanogens	
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-28
$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$	-135.6
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-139.0
Sulfate-Reducing Bacteria	
$CH_3COO^- + SO_4^{2-} \rightarrow 2HCO_3^- + HS^-$	-47.6
$4H_2 + SO_4^{2-} + H^+ \rightarrow 4H_2O + HS^-$	-152.2

Reactions and free energy changes were compiled from various authors [26, 69, 77, 89].

[a] $\Delta G^{\circ\prime}$ calculated from ΔG_f° values provided in Madigan, et al. [55].

2.2.4. IMPORTANCE OF ANOXIC CONDITIONS AND PAH BIOAVAILABILITY

The microbial community in a soil environment may alter soil structure [9] and make bound PAH more accessible in the aqueous phase either as dissolved PAH or as PAH sorbed to "solubilized" organic matter. Burgess and others found that PCB-colloid interactions were more significant with an increased core depth [16]. Perhaps this effect is due to a microbially mediated decrease in oxidation-reduction potential with increased core depth, which results in a more aqueous-soluble fraction of PAH.

A study by Hambrick, et al., in 1980 reported decreased mineralization of naphthalene in sediments with decreased ORP. However, they also reported increased mineralization rates with increased incubation pH, with the highest mineralization occurring at pH 8.0. They concluded that the hydrocarbon-degrading bacteria of the sediment system were adapted to a higher pH, similar to their natural environment, and so had higher metabolic rates at higher pH.

The microbially mediated reactions presented in Table 2.3 demonstrate the consumption of organic acids, $CO_2(g)$, and H^+, which could result in increased pH. Denitrification and sulfate reduction processes result in an increase in alkalinity, which also results in increased pH [77]. Using Figure 2.2 from Stumm and Morgan [77], the relationship between increase in pH and decreased $CO_2(g)$ and increased alkalinity can be easily observed. Humic and fulvic acids are more soluble in solutions with a basic pH [27]. PAH associated with humic and fulvic acids in soil may become more soluble in the aqueous phase with microbially mediated reduced potentials and subsequent increased pH.

If a compound is more soluble in the aqueous phase it is possibly more mobile and bioavailable. DOM-associated PAH can be more effectively transported through the groundwater and aquatic systems [16]. This increased mobility could cause a more hazardous situation because more components of the environment and human populations could be exposed to the contaminant. However, if DOM-associated-PAH migrate to a location with sufficient oxygen and nutrient requirements, biodegradation may also occur.

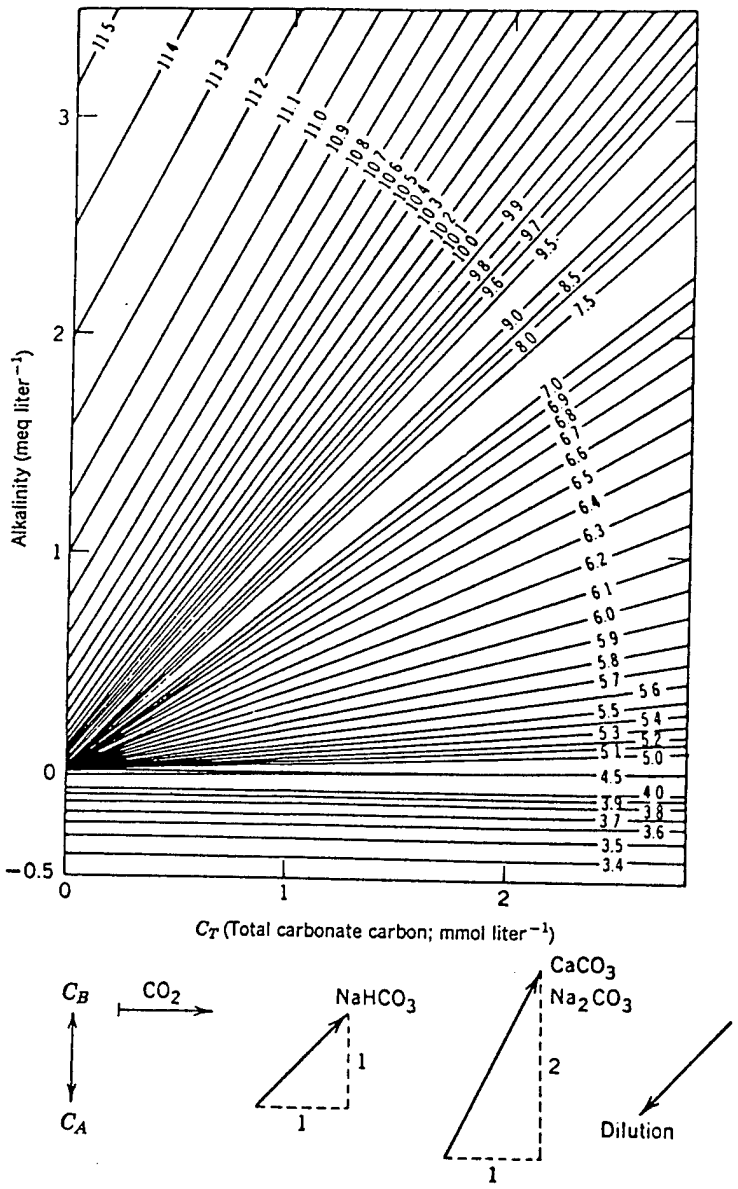

Figure 2.2. pH Contours in Alkalinity versus C_T Diagram. Vectors drawn below chart indicate resulting pH with addition or removal of CO_2, $NaHCO_3$, and $CaCO_3$ or Na_2CO_3. Chart copied from Stumm and Morgan [77].

2.3. MINNESOTA SOIL

2.3.1. REILLY TAR AND CHEMICAL COMPANY SITE HISTORY

The soil used in some of the studies presented in this dissertation was collected from the Reilly Tar and Chemical Corporation Superfund site (St. Louis Park, MN, USA) and is described elsewhere [18, 33]. The historical information that follows I took from the Public Health Assessment prepared by the MN Department of Health and the Agency for Toxic Substances and Disease Registry (http://atsr1.atsdr.cdc.gov:8080/HAC/PHA/reilly-mn/). Reilly Industries, Inc. operated the site from 1917 to 1972, which consisted of an 80-acre plant with a coal tar distillation and creosote wood preserving operation. Coal tar and creosote contain phenolic compounds and PAH. Contamination of the soil and groundwater resulted from the operation of these facilities, above and below ground storage tanks, and a drainage ditch used from 1917 to 1939 for raw wastewater that contained coal tar and creosote. The raw waste emptied into a small marsh until 1940/1941 when Reilly Industries installed a wastewater treatment plant.

Beginning in the early 1930s, coal tar and creosote contamination in groundwater and soil was documented. The Reilly Superfund site sits above six regional aquifers. In 1932, the city drilled its first municipal well approximately one half mile from the Reilly site. This well was closed within weeks of startup due to coal tar taste and odor problems. Residents complained from the 1930s to the 1970s about coal tar contamination in several municipal and private wells. In 1978, wells in St. Louis Park were sampled and analyzed for PAH, for the first time, which led to subsequent closure of seven municipal wells.

In April 1972, the city of St Louis Park purchased the site from Reilly Industries. In June 1973, the site was deeded to the St. Louis Park Housing and Redevelopment Authority. Currently, a large park, three rental condominium and apartment complexes, and commercial and light industrial facilities occupy the location. Local residents continue to use water drawn from municipal wells that contain low levels of PAH contamination (below Drinking Water Criteria as established in the 1986 Consent Decree-Remedial Action Plan). The contaminated subsurface soils on and off-site are considered to be inaccessible to humans due to the vegetative cover, buildings, and pavement. On January 8, 1998, the EPA reported in a news release (www.epa.gov/reg5oopa/news98/98opa005.htm) that the Midwest

Superfund National Priorities List Reilly Tar and Chemical Site had reached "construction completion" in 1997.

2.3.2. IMPLICATIONS OF CURRENT REMEDIATION STRATEGY

The current remediation strategy at the MN site involves the application of topsoil, which may alter the site characteristics and result in a more mobile source of PAH contamination. Strongly reduced conditions are usually established in the leachate plume associated with hazardous waste sites [52, 66]. The topsoil and vegetation, buildings, and pavement may cause anaerobic conditions to prevail in the subsurface. If a decreased redox state alters the soil structure in such a manner as to make the PAH more soluble, significant transport of the PAH contamination from the Reilly site may occur. An understanding of conditions that may influence the fate and sorption of organic contaminants is necessary to delineate the hazards posed to the public and the environment [13].

2.4. OTHER UNCONTAMINATED SOILS USED

I selected the soils used in studies presented in Chapter 4 based on their differences in textural classification, humic and organic carbon content, and cultivation status. None of these three soils, described below, had known or extractable PAH contamination. Some physical characteristics are provided below, a more detailed description is presented in Chapter 4, Table 4.1.

2.4.1. SOUTH DAKOTA (SD)

South Dakota, or SD, soil was collected from an actively cultivated, agricultural area in Winner, SD (Tripp County). The SD soil experienced fertilizer and pesticide application as appropriate in corn and soybean cultivation. SD soil is a clay loam with 6.6% organic carbon and 35% clay.

2.4.2. EDWARDS (ED)

Edwards, or ED, soil was collected from a relatively undisturbed desert location at Edwards Air Force Base, CA (Kern County). ED soil is a sandy loam with 4.9% organic carbon and only 4.5% clay. One of the more significant differences between this soil and others presented in this study is the extremely high sulfur content of ED soil (189 mg/kg soil).

2.4.3. SCHENCK (SK)

Schenck, or SK, soil was collected from the Schenck Research Forest, North Carolina State University, Raleigh, NC (Wake County). SK is a sandy clay loam with 10.9% organic carbon and 20.6% clay.

CHAPTER 3: IMPACT OF IMPOSED ANAEROBIC CONDITIONS AND MICROBIAL ACTIVITY ON AQUEOUS PHASE SOLUBILITY OF POLYCYCLIC AROMATIC HYDROCARBONS FROM SOIL

3.1. INTRODUCTION

The environmental and human health significance of polycyclic aromatic hydrocarbon (PAH) contamination of soils and sediments is well established [1, 2, 21, 64, 85, 91]. In general, the persistence of PAH is increased in soil or sedimentary environments containing natural organic matter [5, 50, 62, 87]. As microorganisms capable of degrading PAH are abundant in the environment, association of PAH via sorption, partitioning or entrapment with soil or sedimentary organic matter is believed to be responsible for decreased microbial availability [15, 46, 88]. Thus, any condition that decreases soil binding of PAH or renders bound-PAH more accessible to microorganisms would theoretically increase the degradation potential. Anaerobiosis is a condition with such potential and its effects on the microbial availability and metabolism of soil-bound PAH are not adequately addressed in the literature.

Soils and sedimentary organic material may frequently experience anaerobic conditions due to the depth in the soil or sedimentary column and, in the case of soil, under flooded hydrological conditions. The thermodynamic sequence of oxidation-reduction reactions mediated by microbes is well established [55, 75]. The microbial community in a soil environment may alter soil structure [9] which could make bound PAH more accessible in the aqueous phase, either as dissolved PAH or as PAH sorbed to "solubilized" organic matter. For example, anaerobic metabolism that employs H_2(aq) as electron donor may cause local pH increases, which leads to enhanced dissolution of soil organic matter. In addition, Burgess and others [16] have found that PCB-colloid interactions are more

significant with increased core depth. These workers and others [16, 30, 38, 61] have observed higher than expected PAH concentrations in an aqueous phase containing soluble natural organic matter, which suggests the existence of PAH-organic soluble complexes. Some authors have shown a decrease in bioavailability to aquatic bluegills, guppies and amphipods due to PAH binding with DOM [29, 48, 57]. However, no reports exist that show altered bioavailability of DOM-associated-PAH to microorganisms under either aerobic or anaerobic conditions.

The first objective of this work was to examine the influence of controlled anaerobic conditions on the aqueous solubility of high molecular weight PAH during a one-year incubation of highly aged, contaminated soil. Our approach was to generate controlled anaerobic conditions using (a) anaerobic headspace or, (b) anaerobic headspace and an oxygen-scavenging complex (titanium(III) citrate) in the aqueous phase or, (c) anaerobic headspace and electron acceptor amendments in the aqueous phase. The anaerobic headspaces used were N_2:CO_2(g) (80:20), N_2(g), or H_2(g). Each treatment profile was compared to an equivalent control with metabolic inhibitors. The second objective of this work was to assess aerobic bioavailability of aged PAH made more soluble under anaerobic conditions. The experimental protocol evaluated the effect on aged PAH solubility due to anaerobic conditions and microbial activity.

3.2. EXPERIMENTAL SECTION

All chemicals and solvents used were of the highest purity available.

3.2.1. SOIL

The soil used in this study was collected from the Reilly Tar and Chemical Company Superfund site (St. Louis Park, MN, USA) and is described elsewhere [18, 33]. This site experienced repeated contamination for over 70 years with wood-treating wastes that contain mixtures of PAH. The total PAH concentration ranges from 231-1571mg-kg^{-1}. This soil will be referred to as "MN soil" throughout the remainder of this paper.

3.2.2. AQUEOUS PHASE PREPARATION OF THE OXYGEN-SCAVENGER AMENDED SAMPLES

The aqueous phase consisted of a titanium(III) citrate complex, an effective oxygen-scavenger, in a mineral medium. Titanium(III) citrate has been used as a reducing agent since it is very effective (E_H = -480 mV) and relatively non-toxic to selected anaerobic microorganisms at high concentrations [42, 44, 92, 94]. The titanium(III) citrate complex was prepared as detailed by Zehnder and Wuhrmann [94]. Titanium(III) chloride (3.75mL of 20% solution) and 0.2M sodium citrate (50mL) were mixed and neutralized with a saturated sodium carbonate solution. The solution was diluted to 2.5mM in the mineral medium. The mineral medium was modified from that used by Mihelcic and Luthy [59]. The salts that follow (in total mg-L^{-1}) were solubilized in de-ionized, high purity water: KH_2PO_4, 8.5; K_2HPO_4, 21.75; $Na_2HPO_4 \cdot 7H_2O$, 33.4; $FeCl_3 \cdot 6H_2O$, 0.25; NH_4Cl, 1.70; $MgSO_4 \cdot 7H_2O$, 22.5; $CaCl_2$, 555; and $NaHCO_3$, 500. Cysteine (50mg-L^{-1}) was also added to the mineral medium.

Aqueous phase preparation was modified from Holdeman's Anaerobe Laboratory Manual [36]. First, the mineral medium was autoclaved, then boiled for 20 minutes prior to adding titanium(III) citrate complex. Formalin (5%) was added as a general metabolic inhibitor to control samples for each type of headspace gas. Next, the aqueous phase (mineral medium plus titanium(III) citrate complex) was cooled while it was degassed with the appropriate headspace gas for each microcosm, either H_2(g), N_2(g), or N_2:CO_2(g) (80:20). The gases were passed over hot copper filings at 300°C to remove traces of oxygen. This process ensured not only that the medium was oxygen free, but that the anaerobic gas present related to the specific treatment condition desired. The "initial" ORP (oxidation-reduction potential), or E_H of the aqueous phases prior to addition to serum vials that contained soil were –91.0mV, -227.7mV, and –227.7mV for N_2:CO_2(g), N_2(g), and H_2(g), respectively, at pH 7.0-7.4. E_H is the measured ORP (E_{meas}) minus the Ag/AgCl reference electrode potential (-222.3mV). Samples treated with this aqueous phase will be referred to as the "Ti-citrate" samples hereafter.

3.2.3. AQUEOUS PHASE PREPARATION OF ELECTRON ACCEPTOR AMENDED SAMPLES

After autoclaving, de-ionized water was amended with one of the following: $NaNO_3$ (4.25g-L^{-1}), Na_2SO_4 (7.10g-L^{-1}), formalin (5%), bromoethanesulfonic acid (BES) (10.55 g-L^{-1}), Na_2SO_4 (7.10g-L^{-1}) and Na_2MoO_4-$2H_2O$ (4.84g-L^{-1}), or $NaNO_3$ (4.25g-L^{-1}) and formalin (5%). BES is a specific inhibitor of methanogens [39, 95], molybdate is a specific inhibitor of sulfate reducing microorganisms [93], and formalin is a general metabolic inhibitor. The pH of each solution, and de-ionized water alone, was adjusted to 7.0 ± 0.1 with NaOH or HCl, then filter sterilized (0.2μm). Samples with de-ionized water were used to assess the effects of imposed anaerobic conditions generated by anaerobic headspace gas only, with no aqueous phase amendments.

3.2.4. MICROCOSMS

Approximately 3g MN soil was added to each 50mL serum vial. For Ti-citrate, the prepared anaerobic aqueous phase (30mL) was added through a needle transfer to stoppered serum vials that contained degassed soil samples. For electron acceptor amended samples, the prepared aqueous phase (30mL) was aseptically added to soil samples. The serum vials were sealed with butyl rubber stoppers. The headspaces were replaced with 5psi(g) or 15psi(g) N_2:CO_2(g), or N_2(g), or H_2(g), for Ti-citrate or electron acceptor samples, respectively. The electron acceptor study and headspace study also compared anaerobic to aerobic conditions. Aerobic headspaces of electron acceptor samples and water samples were replaced monthly by purging with 120mL air. Samples were incubated at 32°C in the dark and shaken monthly over the course of the 365-day study.

3.2.5. ANAEROBIC TO AEROBIC SWITCH

After 181 days, the original anaerobic headspace of some water or electron acceptor samples was purged with air. The 181-day incubation time was selected since Ti-citrate samples showed reproducible amounts of extractable aqueous phase PAH after 149 days incubation. The anaerobic to aerobic headspace (An/Air) samples were incubated 102 days under aerobic conditions. Aerobic headspaces were replaced monthly, after the switch, by purging with 120mL air. Samples were shaken weekly to maintain aerobic conditions.

To confirm aerobic mineralization of PAH, MN soil was incubated with 100 mg-kg^{-1} freshly added pyrene and [4,5,9,10-^{14}C]pyrene (>98% purity, specific activity = 32mCi-mmol^{-1}, Sigma Chemical Company, St. Louis, MO). Microcosms were established and harvested as previously described. In addition, aerobic headspaces were replaced every 30 days to maintain aerobic conditions and collect $^{14}CO_2$ produced. Headspaces were purged with 120mL air, and $^{14}CO_2$ trapped in a scintillation vial that contained 8mL Carbon-14 Cocktail (R.J. Harvey Instrument Corporation, Hillsdale, NJ). $^{14}CO_2$ in the cocktail was determined by liquid scintillation counter (LSC) (Packard 1900TR Scintillation counter, Downers Grove, IL). The soil samples were extracted, as described below, and the residual soil oxidized using a Harvey Biological Oxidizer (R.J. Harvey Instrument Corporation, Hillsdale, NJ) to determine the non-extractable [^{14}C]pyrene and metabolites that remained in the soil or microbial biomass.

3.2.6. AEROBIC TO ANAEROBIC SWITCH

After 90 days incubation, the aerobic headspace of some water or electron acceptor samples was switched to 15psi(g) of an anaerobic headspace (N_2:CO_2, or N_2, or H_2). The 90-day incubation time was selected since previous work completed in our laboratory showed a plateau in PAH mineralization and a drastic decrease in extractability of PAH from soil after 90 days. The aerobic to anaerobic headspace (Air/An) samples were incubated 193 days under anaerobic conditions.

3.2.7. MICROCOSM ANALYSIS

Three or four "live" and three formalin-treated samples were harvested at each time-point. The time-points were 0, 43, 149, 202, 272, and 365 days for Ti-citrate samples; and 90, 180, 283, and 365 days for electron acceptor samples. The Air/An and An/Air samples were harvested on day 283. The headspaces of Ti-citrate samples with H_2(g) were replaced with 15psi H_2(g) after 49 days because the initial 43 day time point revealed significant depletion of the initial H_2(g) headspace pressure.

For each time-point, the headspace pressure, oxidation-reduction state, pH, total organic carbon (days 202-365 only for Ti-citrate), electron acceptor concentrations (NO_3^- and SO_4^{2-}, as appropriate), aqueous phase PAH, sorbed PAH, and extractable PAH were

measured. Headspace pressure was measured with a pressure gauge (Cole-Parmer Instrument Company, Vernon Hills, IL). Next, the serum vials were centrifuged at 2500rpm (910g) for 10 minutes on a Marathon 21 K/R centrifuge (Fisher Scientific, Pittsburgh, PA) to separate soil from pore water. The liquid medium was pipetted from the vial for subsequent analysis. Oxidation-reduction potential was determined with an ORP electrode (Cole-Parmer Instrument Company, Vernon Hills, IL). The pH of the aqueous phase was also recorded.

Total organic carbon (TOC) in the aqueous phase was analyzed with a Shimadzu TOC 5000 Total Organic Carbon Analyzer (Kyoto, Japan). Sample collection, storage, handling, and analysis were conducted in accordance with Standard Method #5310B Combustion-Infrared Method of TOC [28]. A 1mL aliquot of the aqueous phase was acidified to pH<2.0 with HCl, diluted and refrigerated until analysis. Only samples without formalin, molybdate, or BES were analyzed for TOC.

A sample of the electron acceptor aqueous phase was analyzed for appropriate electron acceptor concentration (NO_3^- and SO_4^{2-}). Nitrate levels were determined by a Cu-Cd reduction method on a Lachat QuickChem 4000 automated ion analyzer (Zellweger Analytics, Milwaukee, WI). Sulfate analysis was completed by a turbidimetric method in accordance with Standard Method #4500E [28]. There was no detectable depletion of either electron acceptor over the course of this study presumably due to the high concentrations used.

3.2.8. AQUEOUS PHASE PAH ANALYSIS

The PAH in the medium were solvent extracted and quantified by HPLC. A fraction of the aqueous medium (20mL) from each sample was pipetted from each vial to a 40mL EPA glass vial. The aqueous phase was extracted with two volumes of 10mL dichloromethane (DCM), each extraction shaken 48 hours at 32°C in the dark. The extracts were combined and evaporated to dryness, then re-dissolved in 1mL acetonitrile for HPLC analysis. Aqueous phase extracts were analyzed by HPLC [Waters™ 600E System controller and 717 autosampler; Supelco LC-PAH column (250mm x 4.6mm, ID, Bellefonte, PA) elution gradient from 65% acetonitrile/35% 0.1% trifluoroacetic acid to 100% acetonitrile at 20 minutes with a flow of 1 mL-min^{-1}]. PAH parent and products were

detected by fluorescence (Waters 470 Scanning Fluorescence; excitation 336nm, emission 398nm) and UV absorbance (Spectroflow 757 Absorbance Detector, 254 nm, Kratos Analytical). HPLC chromatography data were analyzed with Millennium 2010 software (Millipore Corporation, Milford, MA).

3.2.9. SOIL PAH ANALYSIS

The MN soil from each treated sample was also extracted with 3 volumes of 10mL DCM. The pooled extracts were filtered through a 20mm Whatman glass fiber filter, type A/E, with a glass flame tower and filter apparatus (Fisher Scientific, Pittsburgh, PA). A 2mL aliquot of the DCM was evaporated and the samples re-suspended in 0.33mL DCM and 1.67mL acetonitrile for HPLC analysis. Average quantities of extractable PAH are presented in Table 3.1. The extractable quantities did not change significantly during the course of the study.

3.2.10. METHANOGENIC STUDIES

Microcosms were established to confirm the presence of an active methanogenic community in the MN soil. The aqueous phase consisted of the mineral medium alone or mineral medium with 5% formalin, 50mM formate, or 50mM formate and 50mM BES. Formate is a substrate metabolized by methanogens [43, 76]. The aqueous phase was prepared as described above. None of the methanogenic study samples contained the Ti-citrate complex. MN soil, aqueous phase, and headspaces were purged with N_2:CO_2 (80:20). Samples were incubated at 32°C in the dark and shaken twice a month over the course of the study. Samples were harvested at 90-day intervals with the same analytical procedures described above. In addition, headspace hydrogen and methane gas were analyzed by TCD (120°C injector and detector, 80°C column, 140mA current) with a Shimadzu GC-8A (Kyoto, Japan) and a 1.8m molecular sieve 5A column, 60/80 mesh, He(g) carrier.

3.2.11. FORMALIN SOLVATING STUDIES

To investigate possible solvating effects of formalin, pyrene dissolved in DCM was added to serum vials, and the solvent allowed to evaporate, leaving 93.4μg pyrene adhered

to the glass vial. Water or water with 5% formalin (50mL), adjusted to pH 7, 8, or 10, was added to the vials and agitated for 30 days at 32°C. The samples were centrifuged, and 35mL of the aqueous phase was carefully pipetted from the top, extracted with DCM, and analyzed by HPLC, as described above.

3.3. RESULTS AND DISCUSSION

Hereafter, "aqueous PAH" refers to pyrene, benz[a]anthracene, and benzo[a]pyrene, combined, unless otherwise noted.

3.3.1. EFFECT OF HEADSPACE GAS

The effect of conditions generated by anaerobic headspace gas alone was assessed by microcosms with N_2:CO_2(g), N_2(g), or H_2(g) headspace and water aqueous phase. The amount of PAH extractable from the aqueous phase increased significantly for all anaerobic headspace gases over the first 90 days relative to aerobic headspace controls (data not shown). All anaerobic headspace samples had 40-60μg-L^{-1} aqueous PAH after 90 days through the end of the study, with H_2(g) amended samples having slightly higher PAH concentrations than N_2:CO_2 (g) or N_2(g) by 365 days (Table 3.2 presents day-283). Aerobic samples maintained less than 10μg-L^{-1} aqueous PAH. BES-inhibited controls showed a significantly different trend as compared to uninhibited headspace samples: BES+H_2(g) exhibited the lowest aqueous PAH concentrations of all anaerobic BES treatments (18.8-32.6μg-L^{-1}), and BES+N_2(g) exhibited the highest aqueous PAH (33.6 to 41μg-L^{-1}). Because BES is a methanogenic inhibitor, these results indicate a potential methanogenic influence, discussed later in this paper. However, all formalin treated samples (headspace, oxygen scavenger, and electron acceptor amended) maintained higher aqueous PAH, owing to the solvating effect of formalin, described later in this chapter.

3.3.2. EFFECT OF HEADSPACE GAS AND OXYGEN SCAVENGER

The microcosms with aqueous phase Ti-citrate complex and anaerobic headspace gas were established to observe effects of anaerobic conditions generated by an oxygen-scavenger and anaerobic headspace. The 43-day harvest showed very low concentrations of aqueous phase PAH that were not able to be accurately quantified. At 149 days, until the

end of the study, the H_2(g)+Ti-citrate samples had the highest extractable PAH concentrations, and N_2:CO_2(g)+Ti-citrate the lowest, which exhibited a similar trend to headspace gas without Ti-citrate discussed above. However, the results presented in Figure 3.1 demonstrate that the aqueous PAH with Ti-citrate treatment was significantly higher than the concentrations listed for anaerobic samples generated by headspace only. H_2(g)+Ti-citrate aqueous PAH was four fold greater than H_2(g) alone. For all three headspaces, benz[a]anthracene and benzo[a]pyrene were above the reported aqueous solubility limits presented in Table 3.1, by as much as an order of magnitude for benzo[a]pyrene (data not shown).

3.3.3. EFFECT OF HEADSPACE GAS AND ELECTRON ACCEPTOR

The microcosms with anaerobic headspaces and electron acceptor amendments were generated to assess potential influence of microorganisms that used NO_3^- or SO_4^{2-}. The addition of electron acceptors to the aqueous phase in both the N_2(g) and H_2(g) samples resulted in an increase in aqueous PAH extractability, as compared to the ambient air control (Table 3.2 presents day-283). The N_2:CO_2(g) headspace samples had lower aqueous PAH than N_2(g) and H_2(g) with either NO_3^- or SO_4^{2-} amendment (Figure 3.2, presents SO_4^{2-}). Molybdate-amended metabolic controls were similar to sulfate samples, except that all samples had approximately 50μg-L^{-1} PAH at 90 days, and increased during the study. Only N_2:CO_2(g)+molybdate samples reached significantly greater aqueous PAH concentration as compared to N_2:CO_2(g)+SO_4^{2-} at 365 days. These results indicate an influence by a microorganism or consortium that is not inhibited by molybdate.

3.3.4. EFFECT OF OXIDATION-REDUCTION POTENTIAL AND pH

The aqueous phase PAH results can be partially explained by abiotic factors that relate to the pH and ORP. The system ORP was dependent on the headspace gas: H_2(g) samples had a more reduced potential than N_2(g) and N_2:CO_2(g) samples (data not shown). In agreement with theory [41], pH values were seen to increase with decreased ORP. Decreased E_H and increased pH enhanced the aqueous solubility of PAH as demonstrated by headspace+Ti-citrate data presented in Figure 3.3. The data plotted exclude formalin-treated samples since these did not produce linear results for reasons discussed later in this chapter.

Headspace gas alone and headspace+electron acceptor samples also demonstrated this pH enhanced aqueous solubility of PAH (data not shown). The decreased ORP and increased pH may have altered soil structure and organic matter solubility, and thus increased the dissolved PAH and PAH sorbed to "solubilized" organic matter. Headspace+Ti-citrate data plotted in Figure 3.4 confirm the relationship between increased aqueous PAH and the log total organic carbon. Headspace and headspace+electron acceptor data exhibited a similar trend, however, the relationship between aqueous PAH and TOC was linear instead of logarithmic (data not shown). In addition, headspace+electron acceptor samples reached a high concentration of only 63mg-L^{-1} aqueous phase TOC. A DOM-PAH interaction explains the higher than aqueous solubility PAH concentrations seen for benz[a]anthracene and benzo[a]pyrene, and is supported by results reported by others [16, 30, 38, 61].

Apparent solubility increase (C_w/C_w^{sat}) for each PAH plotted versus PAH molecular weight (Figure 3.5) established that the association with DOM was influenced by molecular weight. Solubility increase was seen to track directly with hydrophobic character of the PAH regardless of the loading of the PAH (Table 3.1) in the soil of the contaminated site. This I regard as strong evidence of aqueous phase partitioning of the released PAH into soluble organic matter. This was most evident in the H_2(g) headspace samples.

3.3.5. INFLUENCE OF POSSIBLE METHANOGENIC MICROBIAL ACTIVITY

The headspace+Ti-citrate, headspace alone, and headspace+BES data are consistent with a methanogenic influence that enhanced the aqueous solubility of PAH. Methanogens convert organic acids, HCO_3^-, and H_2(aq) to CH_4(g) and water in metabolic processes that consume H^+. In the microcosms prepared with Ti-citrate, the potential methanogenic substrates were excess citrate (approximately 128mg-L^{-1}), cysteine (50mg-L^{-1}), and HCO_3^- (500mg-L^{-1}). These substrates in combination with the H_2(g) headspace may have stimulated the growth of a methanogenic consortium. Citrate effectively chelates charged metal ions from the soil and aqueous phase [77], and may not have been available to microorganisms. However, Perkins, et al. [71], reported conversion of citrate, from Ti-citrate complex, in mixed methanogenic incubations, which used fructose as an electron donor. Cysteine is converted to pyruvate then acetate, while the HCO_3^- can

be used directly by methanogens. An example of the possible anaerobic sequence of reactions is presented below [55, 69]:

$$\text{citrate}^{3-} + 2H_2O \rightarrow \text{formate}^- + 2\text{acetate}^- + HCO_3^- + H^+ \quad (1)$$

$$4\text{formate}^- + 2H+ \rightarrow CH_4 + CO_2 + 2HCO_3^- \quad (2)$$

$$\text{acetate}^- + H_2O \rightarrow CH_4 + HCO_3^- \quad (3)$$

$$4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O \text{ or} \quad (4)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (5)$$

There was a 6.1psi(g) decrease in H_2(g)+Ti-citrate headspace pressure over the course of the study, which indicates an active H_2-consuming group of anaerobes. The magnitude of pH increase (2.1 unit) and ORP decrease (-111mV) coupled with the depleted H_2(g) headspace in these samples constitutes further evidence for the presence of anaerobic microbial activity, for example, methanogenic. In headspace only samples, there was a 3.4psi(g) decrease in H_2(g), a 1.95pH unit increase, and a -164.3mV ORP decrease. With BES treatment (a specific inhibitor of methanogens), extractable aqueous PAH decreased significantly in H_2(g)+BES samples as compared to uninhibited H_2(g) samples. H_2(g)+BES showed a 6.1psi(g) decrease, but only a 0.58 pH unit increase, which indicates a metabolically active, non-methanogenic anaerobic community. These BES results are further evidence of a methanogenic influence that increased aqueous PAH in samples with H_2(g) headspace. All samples with N_2:CO_2(g) headspace exhibited a slight decrease in aqueous pH value (0.1-0.3 unit) owing to CO_2 buffering, and therefore this data set was excluded from presumption of methanogenesis.

Additional microcosms, described above in the Methanogenic Studies section, demonstrated the presence of methanogenic microorganisms present in the MN soil. The data presented in Table 3.3 show higher remaining H_2 with BES treatment, which indicates inhibition of a methanogenic community. The production of CH_4(g), as measured by GC, without BES treatment indicates an active methanogenic population. There was also a corresponding decrease in ORP and increase in pH in samples that contained formate with uninhibited methanogens. The measured pH increased despite the initial 5psi(g) N_2:CO_2(g) headspace. This indicates that the high concentration of formate stimulated a large enough community to significantly decrease the CO_2-HCO_3^- buffering capacity. This pH increase in

a CO_2 buffered system provides further presumptive evidence of the magnitude of pH change that could be induced by methanogenic microorganisms.

There was a significant difference in aqueous PAH and DOM concentrations between headspace and headspace+Ti-citrate samples, presented above. One distinction between these two incubations was the added organic carbon sources in the form of citrate and cysteine and inorganic HCO_3^- in the Ti-citrate aqueous phase. If the methanogens presumed to be active in these samples were carbon-limited, it is attractive to assume that the citrate, cysteine, and HCO_3^- supported a larger or more metabolically active community of methanogens. In addition, the Ti-citrate oxygen scavenger immediately generated an environment more suitable for the obligate anaerobes, compared to the H_2(g) headspace samples in which the trace levels of oxygen that remained in the aqueous phase had to be depleted before methanogenesis could occur. So, methanogens may have been active sooner in H_2(g)+Ti-citrate microcosms than in H_2(g) microcosms. The greater H_2(g) depletion and higher pH is supporting evidence of a greater methanogenic influence in H_2(g)+Ti-citrate samples.

The methanogens that metabolized H_2(aq) alone or H_2(aq) and citrate, cysteine, or HCO_3^-, may have caused an increase in DOM through very large local pH alterations. The measured solution pH, reported above, may not represent the extremely basic pH that a large population of methanogens could potentially generate in microsites of the soil. This localized pH change may result in changes in organic matter structure, which released base-soluble organic matter and organic matter-bound PAH into the aqueous phase. Although this is an attractive interpretation, pH measurement of microsites was not possible in these systems.

3.3.6. INFLUENCE OF POSSIBLE NITRATE-REDUCING MICROBIAL ACTIVITY

The NO_3^--amended microcosms demonstrated a potential influence by nitrate reducing bacteria on increased aqueous PAH. N_2(g)+NO_3^- or H_2(g)+NO_3^- had higher aqueous PAH than N_2(g) or H_2(g) headspace only samples (Table 3.2 lists day-283 results). Dissimilatory nitrate reduction results in the consumption of H^+, thus increased pH. Many chemolithotrophic organisms also possess denitrification capacity, and use H_2(aq) as an electron donor producing N_2O [67, 68, 83]. The 4.2psi(g) decrease in H_2(g) and 2.48 unit

pH increase is evidence that chemolithotrophic organisms were also active in these samples. $H_2(g)+NO_3^-$ samples had the highest pH and most reduced ORP (E_H = -437.7mV) of all samples presented in this study. The increase in aqueous PAH at day-283, and extremely negative ORP further suggests a combined activity of nitrate reducing microorganisms and methanogens. Methanogens require very reduced potentials (E_H < -400mV) and ammonium ions for their nitrogen requirements [69]. It is attractive to assume that the ammonium produced by dissimilatory nitrate reduction may have provided additional nutrients to methanogens. The specific microorganisms present in these microcosms were not identified.

3.3.7. INFLUENCE OF POSSIBLE SULFATE-REDUCING MICROBIAL ACTIVITY

The SO_4^{2-} amended microcosms demonstrated an influence by SRB on aqueous phase PAH solubility. Sulfate treatment resulted in the highest DOM (data not shown) and generally higher aqueous PAH concentrations of all headspace+electron acceptor samples by 365 days (Table 3.2 lists day-283 results). Sulfate reducing bacteria (SRB) have high affinities for H_2(aq) and convert H_2(aq) and sulfate to HS^-, which consumes H^+. The 3.7psi(g) decrease in H_2(g) and 1.7 unit pH increase suggests an active community of SRB. SRB metabolic processes are thermodynamically favored [55], and SRB therefore out-compete methanogens for electron donors as long as sulfate is present [69]. Sulfate samples treated with molybdate, a specific inhibitor of SRB, resulted in lower aqueous PAH concentrations for H_2 headspace samples, which demonstrates the significant influence by SRB on aqueous PAH. The 2.0 pH unit increase and depletion of H_2(g), 3.7psi(g), I regard as evidence of a microbial community which was active in the absence of SRB.

3.3.8. EFFECT OF ANAEROBIC TO AEROBIC INCUBATION

The second objective, accomplished by An/Air treatment, was to assess aerobic bioavailability of aged PAH made more soluble under anaerobic conditions. There was a significant decrease in extractable aqueous PAH, for most An/Air samples, as compared to constant anaerobic samples (Table 3.3). The decrease may have been due to aerobic PAH degradation, sequestration in soil, PAH-DOM precipitation, or aerobic DOM-degradation. The MN soil amended with [^{14}C]pyrene showed that after 90 days, approximately 10% of

the radiolabel was converted to $^{14}CO_2$, indicating a decrease in aqueous PAH due to biodegradation. Over 7% of the [^{14}C]pyrene was bound as non-extractable soil residues after 90 days, which suggests a decrease in aqueous PAH due to sequestration. The decrease in pH from the more basic anaerobic conditions of N_2(g) and H_2(g) samples suggests that humic acids and DOM-PAH precipitated. All An/Air samples showed a 30-50mg-L^{-1} decrease in TOC as compared to constant anaerobic samples. In addition, microorganisms capable of using soil organic carbon as a carbon source presumably decrease DOM [60, 70], thus accounting for some DOM decrease.

A smaller change in extractable aqueous phase PAH was seen in H_2/Air+NO_3^- samples as compared to constant H_2(g) samples (Table 3.2). In these samples, the extremely reduced environment (E_H = -437.7mV), pH of 9.5, and potential NH_4^+ concentrations after 180 days under anaerobic conditions may have prevented significant aerobic metabolism or transformation of PAH. Although the pH of these samples decreased from 9.5 to 7.9 under aerobic conditions, these samples had the highest final pH of all An/Air samples. These results indicate that if aerobic microorganisms were active, they had not recovered to the degree of other samples and therefore did not metabolize or sequester PAH.

A notable increase in extractable aqueous PAH was seen in N_2/Air+ and H_2/Air+molybdate samples (Table 3.2). Methanogens reduce sulfate and use SH^- to form cysteine and other amino acids [55]. Cysteine has been shown to have bactericidal effects when exposed to atmospheric oxygen due to hydrogen peroxide formation [17]. Neither cysteine nor hydrogen peroxide were analyzed in these samples. It is possible that cysteine or some other methanogenic metabolite may have caused a toxic environment, and prevented metabolism under aerobic conditions.

3.3.9. EFFECT OF AEROBIC TO ANAEROBIC INCUBATION

The Air/An treatment was intended to determine if aerobic conditions and microorganisms alter the soil and PAH, and affect the PAH solubility when the system is converted to anaerobic conditions. PAH mobilization, as seen in other anaerobic incubations presented in this chapter, did not occur in Air/An systems (Table 3.2), despite similar increases in pH and DOM concentrations. As stated in the An/Air section, there were multiple plausible explanations for a decrease in aqueous PAH under aerobic

conditions. The Air/An results suggest that aerobic microorganisms altered soil structure, or mineralized/sequestered PAH such that the PAH did not associate with the DOM that became soluble when anaerobic conditions were imposed.

3.3.10. SOLVATING EFFECT OF FORMALIN

The aqueous phase results of the formalin-treated samples did not show the same linear trends, with relation to ORP and pH, as the untreated (live) samples. The formalin solvating study showed that the formalin treatment resulted in 15% greater aqueous concentrations of pyrene than those with water. Formalin solutions contain 37% formaldehyde, 10% methanol, and 53% inert ingredients. The methanol component may account for the solvating effect of formalin. The formalin data also reflected the influence of the buffer in the formalin solution, which maintained the pH between 6.0-7.4, regardless of the headspace gas. Despite the constant pH, the oxidation-reduction potential was similar to live samples. This reduced potential may result from the presence of methanol or the breakdown of formalin, which resulted in $H_2(g)$. Detectable $H_2(g)$ peaks were observed in the analysis of the formalin treated sample of the Methanogenic Studies, however, these were below the lowest standard (1%), and could not be accurately quantified (Table 3.3).

3.4. CONCLUSIONS

The proposed ORP-related pH and microbial influences on organic material, and subsequent solubilization of PAH, were consistent with all experimental observations. This study demonstrated significant aqueous phase PAH effects due to controlled anaerobic incubation. Methanogens were seen to have the greatest effect on aqueous PAH solubility, however, SRB may have a greater influence than methanogens when a suitable carbon source for methanogens is limited. PAH made more soluble under anaerobic conditions were available to be degraded or transformed under aerobic conditions. This study emphasizes the importance of considering the microbial community in a system since they will influence PAH directly or indirectly through pH and DOM alteration. These anaerobic influences may have important implications on bioavailability, especially if organic matter associated-PAH migrate to a location with sufficient oxygen and nutrient requirements for biodegradation.

Table 3.1. Physical Characteristics of Some High Molecular Weight PAH in MN Soil.

Compound	M.W. ($g\text{-}mole^{-1}$)[a]	Log K_{ow}[a]	Aqueous Solubility ($\mu g\text{-}L^{-1}$)[a]	Average PAH Extractable from MN soil ($mg\text{-}kg^{-1}$)
Fluoranthene	202.3	5.22	260	105.3 ± 11.7
Pyrene	202.3	5.18	130	155.9 ± 19.6
Benz[a]anthracene	228.3	5.91	11.0	49.4 ± 6.5
Benzo[a]pyrene	252.3	6.04	3.8	138.8 ± 12.4

[a]Adapted from [53].

Table 3.2. Aqueous Phase PAH Concentrations at 283-Days of Incubation.

	Constant Headspaces				Anaerobic to Aerobic Switch			Aerobic to Anaerobic Switch		
	Air	N_2:CO_2	N_2	H_2	N_2:CO_2/Air	N_2/Air	H_2/Air	Air/N_2:CO_2	Air/N_2	Air/H_2
Water ("headspace only")	2.4	48.5	55.6	54.0	1.9[a]	2.3[a]	24.9[c]	6.1	6.3	5.5
BES	5.9	32.3	36.9	28.0	2.2[a]	1.4[a]	16.4[c]	6.8	7.4	4.2
Nitrate	1.9	38.8	68.3	80.3	4.1[a]	28.5[c]	60.4[b]	6.4	7.5	10.8
Sulfate	3.4	54.4	67.6	90.8	2.2[a]	13.1[a]	32.2[b]	5.5	6.4	7.1
Molybdate	12.2	55.4	69.8	74.3	12.4[b]	76.5	103.5[b]	9.3	10.8	8.1

Data indicates $\mu g\text{-}L^{-1}$ extractable aqueous phase PAH determined by HPLC. The following letters indicate significant difference (based on two-tailed Student's t test) between the indicated An/Air sample and the corresponding anaerobic only headspace: [a] $p<0.01$; [b] $p<0.02$; [c] $p<0.20$.

Table 3.3. Methanogenic Study Aqueous Phase and Headspace Results.

	Day 0		Day 90			
Sample	pH	E_H (mV)	pH	E_H (mV)	H_2	CH_4
MM[a]	6.7	152.3	7.1	87.5	<1%	<1%
MM + Formalin	6.5	62.3	6.8	43.3	<1%	ND[b]
MM + Formate	6.6	102.3	8.1	-5.00	ND	13.5%
MM + Formate + BES	6.6	62.3	7.7	-65.5	55.9%	ND

[a]MM = mineral medium, [b]ND = none detected. $E_H = E_{meas} + 222.3mV$, based on Ag/Ag-Cl reference electrode. Average H_2 and CH_4 measured in the headspace of triplicate samples.

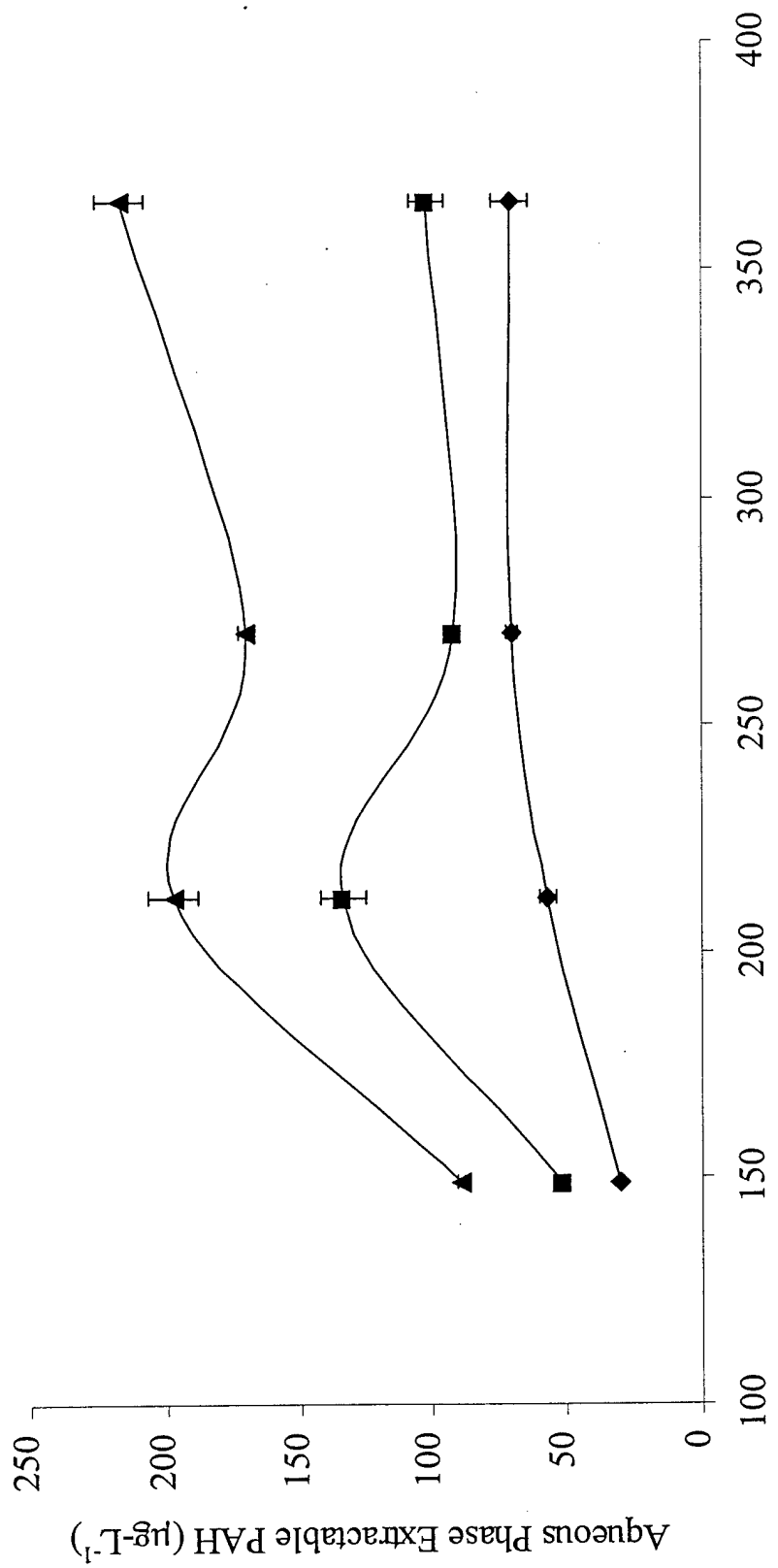

Figure 3.1. Extractable aqueous PAH (pyrene, benz[a]anthracene, and benzo[a]pyrene, combined) in MN soil samples incubated with Ti-citrate. Error bars represent the average deviation from the mean of quadruplicate samples: (◆) N_2:CO_2 (80:20); (■) N_2; and (▲) H_2.

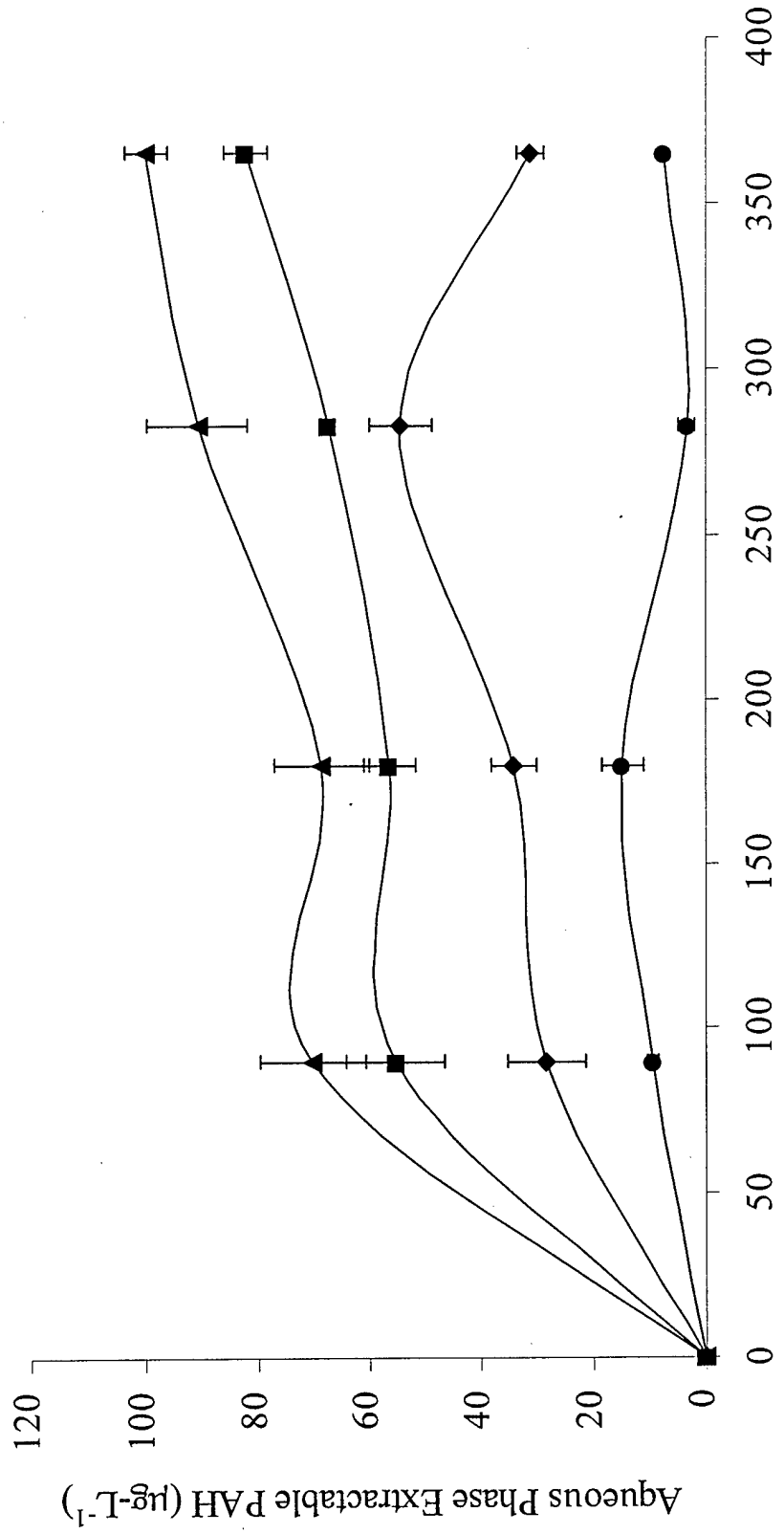

Figure 3.2: Extractable aqueous PAH (pyrene, benz[a]anthracene, and benzo[a]pyrene, combined) in MN soil samples incubated with 7.10g-L^{-1} sulfate. Error bars represent one standard deviation from the mean of triplicate samples: (●) Aerobic; (◆) N_2:CO_2 (80:20); (■) N_2; and (▲) H_2.

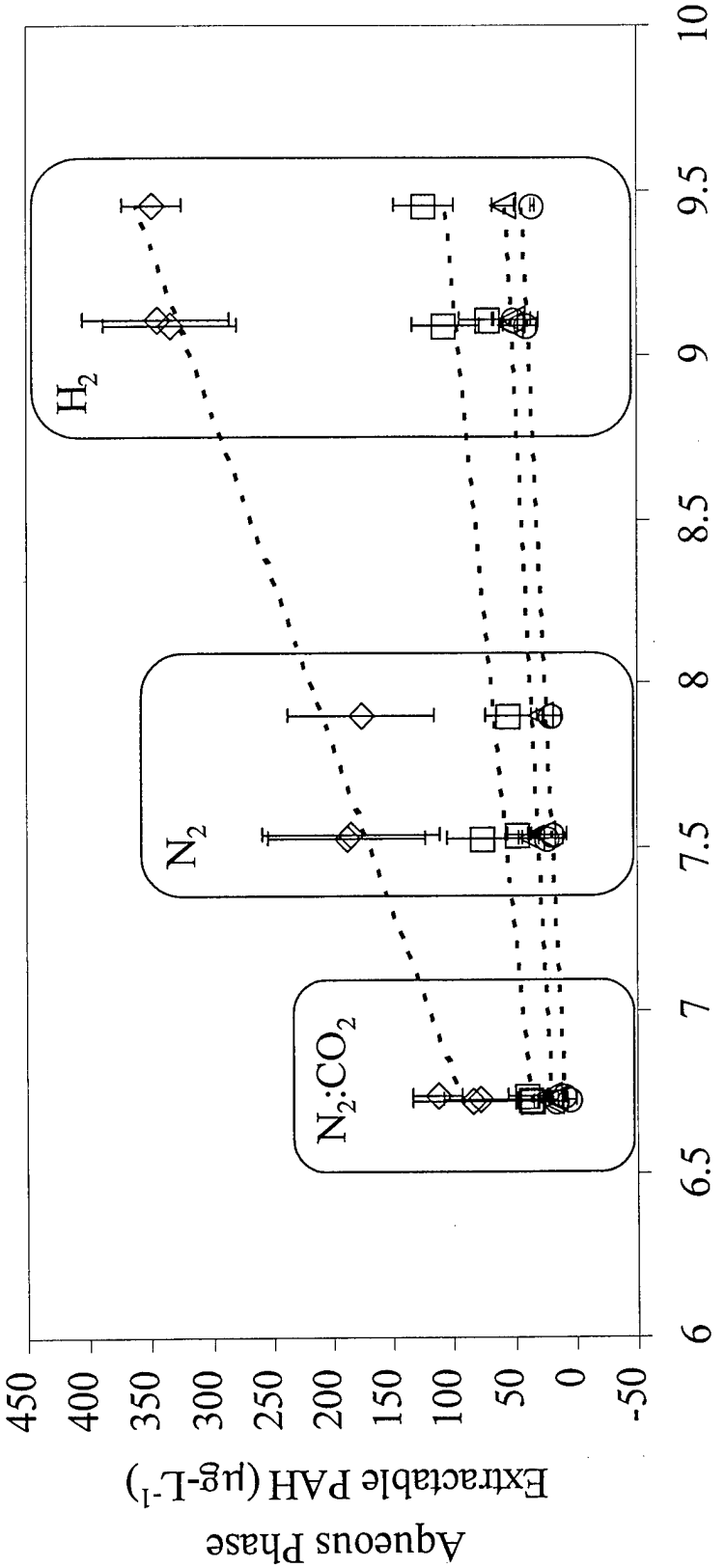

Figure 3.3. Effect of pH on aqueous PAH concentration after 202, 272, and 365-days incubation with Ti-citrate. Rectangles drawn indicate data groupings according to headspace gas indicated. Error bars represent one standard deviation from the mean of quadruplicate samples, r^2 value for each linear regression presented is included after the symbol: (△) fluoranthene, r^2=0.97; (□) pyrene, r^2=0.79; (△) benz[a]anthracene, r^2=0.94; and (○) benzo[a]pyrene, r^2=0.85.

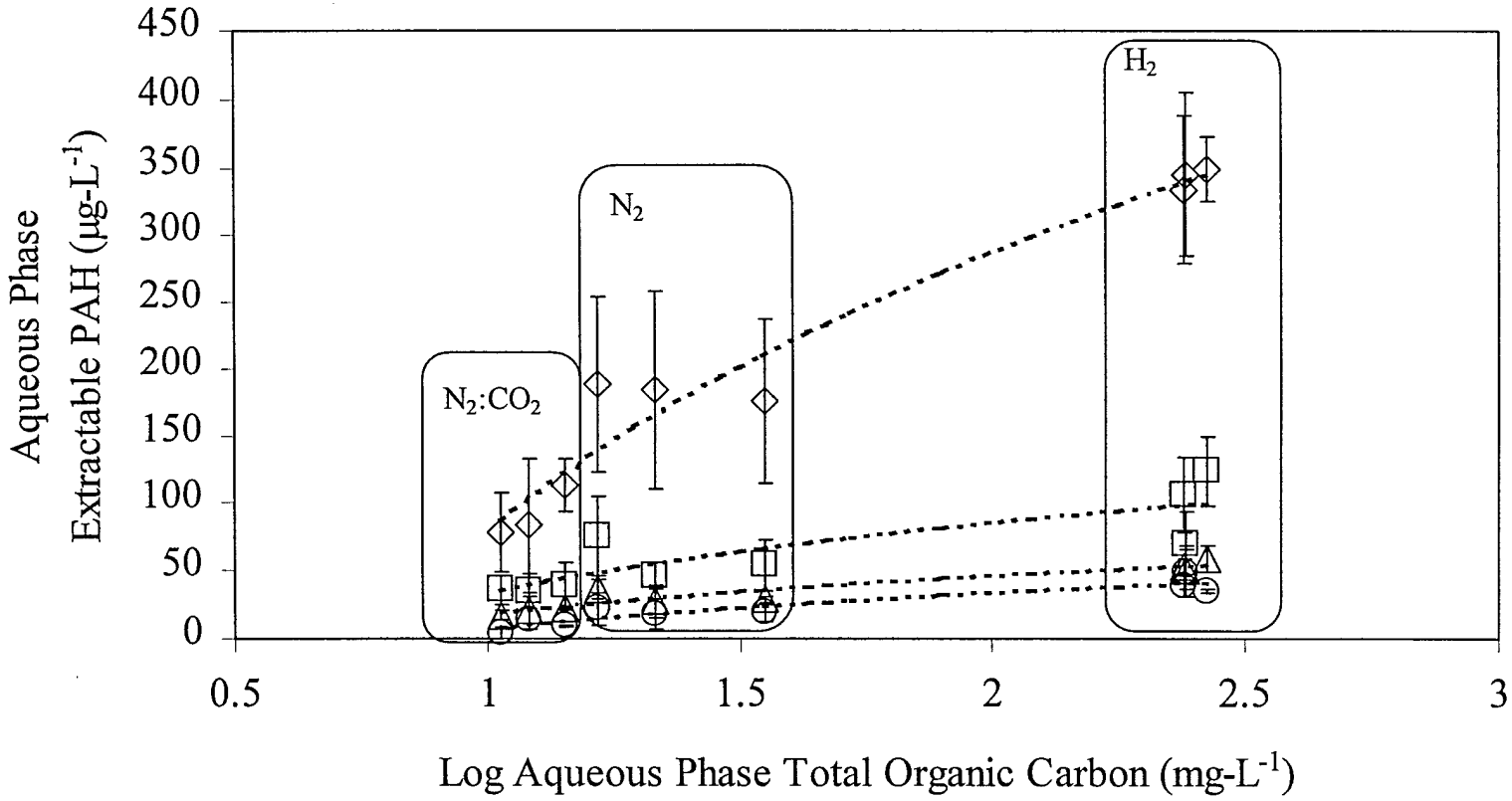

Figure 3.4. Aqueous PAH association with aqueous phase log total organic carbon after 202, 272, and 365-days incubation with Ti-citrate. Rectangles drawn indicate data groupings according to headspace gas indicated. Error bars represent one standard deviation from the mean of quadruplicate samples, r^2 value for each regression presented is included after the symbol: (◇) fluoranthene, r^2=0.95; (□) pyrene, r^2=0.70; (△) benz[a]anthracene, r^2=0.90; and (○) benzo[a]pyrene, r^2=0.86.

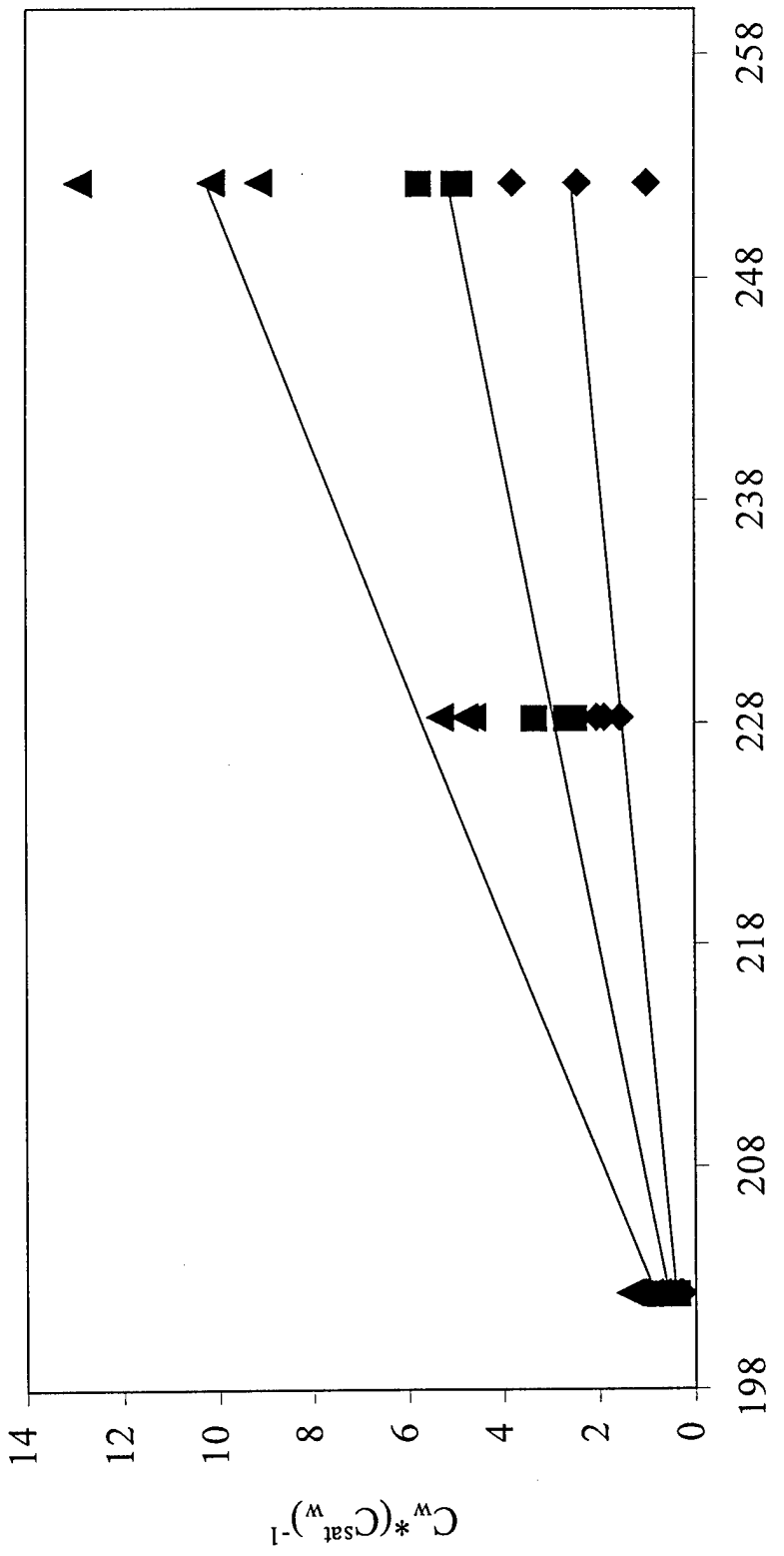

Figure 3.5. Aqueous PAH normalized by aqueous phase solubility versus the PAH molecular weight after 202, 272, and 365-days incubation with Ti-citrate. Each symbol represents the mean of quadruplicate samples, r^2 value for each linear regression presented is included after the symbol: (◆) N_2:CO_2 (80:20), r^2=0.71; (■) N_2, r^2=0.98; and (▲) H_2, r^2=0.94.

CHAPTER 4: MICROBIAL BIOAVAILABILITY OF PYRENE IN THREE LABORATORY-CONTAMINATED SOILS UNDER AEROBIC AND ANAEROBIC CONDITIONS

4.1. INTRODUCTION

Pyrene is a high molecular weight polycyclic aromatic hydrocarbon (PAH). The environmental and human health significance of PAH in contaminated soils and sediments is well established [1, 2, 21, 64, 85, 91]. PAH persistence appears to increase in soil or sedimentary environments that contain natural organic matter [4, 5, 50, 62, 87]. PAH associates with the natural organic matter via a number of processes that can be simplified to sorption, partitioning, or entrapment, thus making less material bioavailable for aerobic microbial transformation [15, 46, 65, 88]. The formation of bound PAH-residues and non-extractable PAH fractions, has been reported in the literature [5, 6, 10, 12, 19, 33, 45]. Some researchers have suggested that microorganisms facilitate the sequestration of xenobiotics in an attempt to reduce the toxicity of a contaminant or its metabolic products [12, 20].

Any condition that enhances microbial metabolic activity, decreases soil binding of PAH, or renders bound-PAH more accessible to microorganisms would theoretically increase the degradation potential. Addition of inorganic nutrients, such as nitrate or sulfate, may stimulate microbial growth and enhance biodegradation of organic compounds [56]. In addition, Manilal and Alexander report phenanthrene mineralization when it is sorbed to muck soil. This indicates that PAH is degraded even in a sorbed state in the presence of specific soil organic matter characteristics [56]. Anaerobic conditions may also enhance bioavailability of PAH, and this subject is not adequately addressed in the literature. Data presented in Chapter 3 demonstrate that soil-bound PAH are mobilized under reduced conditions as dissolved organic matter (DOM)-associated PAH.

Under anaerobic conditions, a terminal electron acceptor may induce the most thermodynamically favored consortia of microorganisms to dominate a system [55]. These microorganisms mediate a specific oxidation-reduction potential [63], and potentially alter the chemical and physical characteristics of the soil [9]. Anaerobic metabolism may cause local pH increases, which leads to increased dissolution of soil organic matter. The effect of electron acceptor selected consortia of anaerobes associated with PAH-contaminated soil is also addressed in Chapter 3.

The specific objectives of this work were to examine the influence of (a) aerobic conditions and inorganic nutrient addition, and (b) anaerobic conditions and electron acceptor amendment, on the bioavailability of pyrene in three distinctly different soils during a one-year incubation. Soils were selected based on their differences in textural classification, humic and organic carbon content, and cultivation status. Three previously uncontaminated soils were aged with pyrene and [^{14}C]pyrene for 65 days, then incubated with water, NO_3^-, or SO_4^{2-} under aerobic or anaerobic conditions for one year. Anaerobic conditions were generated with the electron acceptors and N_2:CO_2(g) headspace. Each treatment profile was compared to an equivalent control with metabolic inhibitors. The experimental protocol evaluated (a) the effect of inorganic nutrient addition under aerobic conditions on 65-day aged pyrene bioavailability and biodegradation, and (b) the effect of anaerobic conditions and metabolically active anaerobic microbial communities on pyrene associations with soil.

4.2. METHODS

All chemicals and solvents used were of the highest purity available.

4.2.1. SOIL SAMPLING AND DESCRIPTION

The three soils used in this study were obtained from surface locations with no known or measured PAH contamination. The first soil (ED) was collected from a relatively undisturbed desert location at Edwards Air Force Base, CA. The second soil (SD) was collected from an actively cultivated, agricultural area in Winner, SD. The SD soil experienced fertilizer and pesticide application as appropriate in corn and soybean cultivation. The third soil (SK) was collected from the Schenck Research Forest, North

Carolina State University, Raleigh, NC. Soil samples were stored at 4°C in the dark until use.

Physical characteristics for each soil are listed in Table 4.1. The soils used were analyzed by the North Carolina Department of Agriculture and Consumer Services Agronomic Division, Raleigh. The soil class is based on the humic matter percent and weight/volume ratio. Humic matter percent represents the amount of soil organic matter (humic and fulvic acid components) that is soluble in a sodium hydroxide solution. This calculation does not account for the humin that is left in the soil and is therefore not representative of percent organic carbon. The Agronomic Division used the Mehlich-3 extractant procedure to determine the major nutrients and micronutrients in the soil [58]. The percent of cation exchange capacity (CEC) occupied by bases is principally comprised of calcium, magnesium, and potassium [84]. The percent organic carbon was determined in our laboratory by oxidizing the soils in a muffle furnace at 500°C for 4 hours. The pH was also analyzed in our laboratory by the pH-H_2O procedure [40]. The percent sand, silt, and clay were determined in our laboratory using the Bouyoucos Method [14], and textural classification determined graphically from a chart in Thompson [82].

4.2.2. PAH APPLICATION AND AGING IN SOIL

To prepare the soil aged with pyrene, 400 g of each soil (2 mm sieved) was autoclaved three times for 1 hour (days 1, 2, and 4). The soil was aseptically transferred to a jar and autoclaved, de-ionized water was added (400mL). Pyrene and [4,5,9,10-^{14}C]pyrene dissolved in methanol was added to the soil and water, final concentrations were desired to be 100 mg-kg^{-1} pyrene and 600,000 Bq-g^{-1}. Pyrene and [^{14}C]pyrene (>98% purity, specific activity = 1.2 x 10^{9} Bq-mmol^{-1}) were purchased from Sigma Chemical Company (St. Louis, MO). Jars were stored at 32°C in the dark, and vigorously shaken for 1 hour, 3 times a week, for 65 days. At the end of the aging period, the water was carefully decanted, and a portion of the soil was dried for 5 days. The soil was then mixed with "live" untreated soil in a ratio of 1:1 to restore native microbial communities. Aliquots of mixed soil were combusted to determine the initial amount of radiolabeled pyrene present, and ensure uniform mixing of pyrene and soil, with a Harvey Biological Oxidizer (R.J. Harvey Instrument Corporation, Hillsdale NJ).

4.2.3. AQUEOUS PHASE AND MICROCOSM PREPARATION

The aqueous phase consisted of autoclaved de-ionized water alone, or with $NaNO_3$ (4.25 g-L^{-1}) or Na_2SO_4 (7.10 g-L^{-1}). Metabolically inhibited samples contained 5% formalin for water or NO_3^- samples, or molybdate (4.84 g-L^{-1}) in SO_4^{2-} samples. Molybdate is a specific inhibitor of sulfate-reducing bacteria (SRB) [93], and formalin is a general metabolic inhibitor. The media were adjusted to a pH of approximately 7.0 ± 0.1 with HCl or NaOH, then filter sterilized (0.2 μm).

Each microcosm consisted of 4g mixed soil and 30mL aqueous phase added aseptically to a 50mL serum vial and sealed with butyl rubber stoppers. The headspace of the anaerobic vials was purged with N_2:CO_2 (80:20), to a final headspace pressure of 5 psi(g). Aerobic headspaces were replaced monthly by purging with 120mL air, as described below. Samples were incubated in the dark at 32°C, and shaken weekly. A detailed diagram of the sample set-up is presented in Figure 4.1.

4.2.4. HEADSPACE ANALYSIS

Aerobic headspaces of live samples were replaced approximately every 30 days to maintain aerobic conditions and collect $^{14}CO_2$ generated. Headspaces were purged with 120mL air into a scintillation vial that contained 8mL Carbon-14 Cocktail (R.J. Harvey Instrument Corporation, Hillsdale, NJ) to trap $^{14}CO_2$ (Figure 4.1). The purge procedure did include soluble forms of inorganic $^{14}CO_2$, however, since the pH was consistently neutral, I assumed the difference was insignificant. The headspace samples were analyzed on a liquid scintillation counter (LSC) (Packard 1900TR Scintillation counter, Downers Grove, IL).

4.2.5. MICROCOSM ANALYSIS

Three live samples for each treatment condition were harvested at 90, 180, 270, and 365 days. Three metabolically inhibited samples for each treatment conditions were harvested at 180 and 365 days. For each time-point, the headspace pressure, $^{14}CO_2$, oxidation-reduction state, pH, electron acceptor concentration (NO_3^- and SO_4^{2-}, as appropriate), aqueous phase PAH, extractable PAH, and sorbed PAH were measured. First, the headspace pressure was measured with a pressure gauge (Cole-Parmer Instrument

Company, Vernon Hills, IL). Next, the serum vials were centrifuged at 2500 rpm (910 X G) for 10 minutes on a Marathon 21 K/R centrifuge (Fisher Scientific, Pittsburgh, PA) to separate soil from pore water. The $^{14}CO_2$ in the headspace was purged as described above and analyzed by LSC. The liquid medium was pipetted from the vial for subsequent analysis. Oxidation-reduction potential was determined with an ORP electrode (Cole-Parmer Instrument Company, Vernon Hills, IL). The pH of the aqueous phase was also recorded. An aliquot of the aqueous phase was analyzed for radiolabel metabolites and parent compounds on LSC.

A sample of the aqueous phase pore water was analyzed for appropriate electron acceptor concentration (NO_3^- and SO_4^{2-}). Nitrate levels were determined by a Cu-Cd reduction method on a Lachat QuickChem 4000 automated ion analyzer (Zellweger Analytics, Milwaukee, WI). Sulfate analysis was completed according to a turbidimetric method described in Standard Method #4500E [28].

The soil from each sample was extracted 3 times (24 hours each) with 10mL dichloromethane (DCM). The pooled extracts were filtered through a 20mm Whatman glass fiber filter, type A/E, using a glass flame tower and filter apparatus (Fisher Scientific, Pittsburgh, PA). A sample of the soil extract was analyzed by LSC to determine extractable [^{14}C]pyrene.

Soil samples were oxidized, after DCM extraction, to determine the non-extractable [^{14}C]pyrene that remained in the soil. Samples were air-dried, combusted using a Harvey Biological Oxidizer (R.J. Harvey Instrument Corporation, Hillsdale NJ), and [^{14}C]pyrene, as $^{14}CO_2$, quantified by LSC.

4.3. RESULTS AND DISCUSSION

4.3.1. AEROBIC ED, SD, AND SK MICROCOSMS: MINERALIZATION

In aerobic treatments, there were sufficient microorganisms present in the native soil to mineralize a significant fraction of [^{14}C]pyrene in ED and SD samples, but not SK. After one year, ED samples mineralized up to 82%, SD samples mineralized up to 58%, while SK samples had only sporadic mineralization (Figure 4.3, ED samples shown). No metabolically inhibited samples with formalin had detectable levels of $^{14}CO_2$.

NO_3^- amendment caused a significantly higher rate of metabolism ($p<0.10$) as compared to water only in both SD and ED samples through 159-days of incubation. However, this higher rate moderated by 365-days. Over the course of the study, SD microcosms depleted 13.3 $\mu g\text{-}L^{-1}$ NO_3^-, while ED microcosms depleted 252 $\mu g\text{-}L^{-1}$ NO_3^-. Microorganisms assimilate NO_3^- under aerobic conditions [55], and this may have acted as a nutrient for microorganisms.

ED samples amended with molybdate showed a lower mineralization rate as compared to SO_4^{2-} treated samples by at least one standard deviation from the mean. Molybdate inhibits sulfate-reducing bacteria, and may have affected some microorganisms in ED samples. ED soil had very high initial concentrations of sulfur (Table 4.1) and 1.8 $g\text{-}L^{-1}$ as SO_4^{2-}. Aerobic samples with molybdate depleted 60 $mg\text{-}L^{-1}$ less SO_4^{2-} as compared to uninhibited ED microcosms. There was no significant difference between SO_4^{2-} or molybdate amended SD samples.

4.3.2. AEROBIC ED, SD, AND SK: [^{14}C]PYRENE DISTRIBUTION IN SAMPLES

Significant differences were observed in the distribution of label during aerobic utilization for ED and SD soils. SD microorganisms mineralized pyrene in the solution phase, which caused a decrease in the extractable fraction as mineralization proceeded (Figure 4.4 presents SD samples with water aqueous phase). It is noteworthy that the mass balance in isotope distribution at times approaching one year suggests that extended mineralization is accompanied by conversion of non-extractable material into a bioavailable extractable or aqueous phase. I also note that NO_3^- amendment in SD soil showed a significantly higher ($p<0.05$) rate of conversion of non-extractable to extractable label midway through the utilization study. NO_3^- amended samples had 41% non-extractable [^{14}C]pyrene as compared to 61.7% in water and 58.9% in SO_4^{2-}-treated samples at 180 days. However, the value of the non-extractable fraction in NO_3^--amended samples at one year was equivalent to water only samples (approximately 33%). Formalin inhibited water and NO_3^- SD samples maintained an approximately 80% level of non-extractable radiolabel. For aged pyrene in SD soil, a one year incubation proceeded to a significant degree in terms

of conversion of pyrene to CO_2, even when a high percent of the initial pyrene was firmly bound to soil in the non-extractable fraction.

For ED soils (Figure 4.5 presents water aqueous phase ED samples), as mineralization proceeded, it appears that sufficient label was present in the extractable compartment to accommodate the needs of the microorganisms. These results are undoubtedly due to the fact that a larger portion of the extractable phase was bioavailable in ED soil. Consequently, there was not a significant decrease in the non-extractable fraction.

Although SK samples demonstrated only sporadic mineralization, we nevertheless observed a significant redistribution of label from extractable to non-extractable over the year incubation. For example, the non-extractable fraction rose from 12.5% to 70% and extractable fraction decreased from 80% to 25%.

The comparatively high distribution of label in the extractable fraction in ED samples was probably due to its significantly lower humic content (reference Table 4.1) as compared to SD. However, the SK soil also had high humic content and yet most of the added label was initially extractable, at a similar level to ED soil. Less of the starting label may have been extractable in the SD soil compared to ED and SK because of the significantly higher clay content in SD soil. In soils with low organic matter content, clays have been reported to compete with humics for binding contaminant [12].

4.3.3. ANAEROBIC ED, SD, AND SK MICROCOSMS

No mineralization of pyrene to $^{14}CO_2$ was observed in any soils under anaerobic conditions.

After 180 days there was evidence of anaerobic microbial activity through NO_3^- and SO_4^{2-} utilization and a concurrent decrease in the non-extractable fraction and an increase in the extractable fraction. All ED and SK samples, live and metabolically inhibited, began with high amounts of extractable pyrene (approximately 80%) that decreased to what appeared to be an equilibrium or stable concentration after 180 days (SK $\simeq$ 25%, ED $\simeq$ 40%). However, the last time-point showed an increase in extractable pyrene from this apparent equilibrium for NO_3^- and SO_4^{2-} samples (Figure 4.6 presents SK, SO_4^{2-}). In ED soils treated with NO_3^- and SO_4^{2-}, the extractable concentrations increased from the 40%

apparent equilibrium at 180 days to 53% of total added radiolabel by 365 days. The water-treated samples and all metabolically inhibited controls for ED and SK samples showed no change from the apparent equilibrium established at 180 days.

A significantly different pyrene distribution was observed in SD samples. SD samples showed a decrease in extractable [^{14}C]pyrene from 26.1% to less than 10% in all treatments. There was a corresponding increase in the non-extractable pool of SD soil from 73.7% to approximately 82.2% in all treatments, which was maintained from 180 days until the end of the study.

The increase in extractable pyrene in NO_3^- and SO_4^{2-} ED and SK samples may indicate microbially mediated changes in the soil structure and sequestration of pyrene. Microorganisms reducing NO_3^- and SO_4^{2-} may cause changes in soil structure due to large localized pH changes in microsites of the soil, as addressed previously in Chapter 3. Dissimilatory nitrate reduction results in the consumption of H^+, and thus an increased pH. All live samples had a detectable depletion of aqueous phase NO_3^- (ED = 0.53 mg N-L^{-1}, SD = 0.26 mg N-L^{-1}, SK = 1.1 mg N-L^{-1}), evidence of microbially mediated NO_3^- reduction. Sulfate treatment resulted in the greatest change in extractable pyrene in ED and SK microcosms as compared to molybdate metabolically inhibited controls. SRB convert H_2(aq) and sulfate to HS^-, consuming H^+. All samples, excluding SD and SK with molybdate, depleted SO_4^{2-} (ED = 1.7 g-L^{-1}, ED+molybdate = 0.7 g-L^{-1}, SD = 1.6 g-L^{-1}, SK = 3.6 g-L^{-1}) indicating active SRB. However, the anaerobic samples had a 20% CO_2 headspace that acted as a buffer, which maintained the pH of the microcosms near neutrality. According to Jacob, theory dictates that pH values increase with decreased ORP [41]. We observed a significant decrease in ORP in all anaerobic ED, SD, and SK samples (Table 4.2), as compared to aerobic treatment, which indicates a potential increase in pH that was masked by CO_2 buffering.

Sulfate amendment resulted in notable changes in the physical appearance of all microcosms. The soil in SD and SK aerobic and anaerobic samples treated with SO_4^{2-} exhibited notable swelling. After centrifugation, 25-27mL of the aqueous phase could be pipetted from the microcosms for most soils. In SD and SK SO_4^{2-} treated sample, only 15-20mL of the aqueous phase was removable due to the soil swelling. The soil of ED, SD, and SK SO_4^{2-} microcosms became a black color within a month of incubation, evidence of metal

sulfide precipitation, such as FeS(s). This swelling and changes in the inorganic composition of the microcosms indicate another treatment-induced change in the soil that may have enhanced pyrene extractability.

4.4. CONCLUSIONS

This study demonstrated that there were microorganisms in previously uncontaminated soils that were capable of degrading pyrene under aerobic conditions. SD soil samples showed that even when a high percent of the initial aged pyrene is non-extractable, significant mineralization could occur. This study also showed that anaerobic conditions with NO_3^- and SO_4^{2-} notably increased the extractability of pyrene from some soils. These observations may have important implications concerning bioavailability and transport of PAH in the environment.

Table 4.1. Physical Properties of Soils.

	ED	SD	SK
Soil Class	Mineral Soil	Mineral Soil	Mineral Soil
Textural Classification	Sandy Loam	Clay Loam	Sandy Clay Loam
% Organic Carbon	4.90%	6.57%	10.90%
% Humic Matter ($g/100cm^3$ soil)	0.04%	0.76%	0.65%
% Sand	54.85%	36.76%	57.63%
% Silt	40.70%	28.32%	21.78%
% Clay	4.45%	34.92%	20.59%
Weight/Volume of Soil (g/cm^3)	1.27	1.15	1.02
CEC ($meq/100cm^3$ soil)	46.2	34.7	10.1
% CEC occupied by bases	100%	100%	85%
pH^a/ pH^b	7.7^a / 8.1^b	7.8^a / 8.2^b	6.1^a / 6.8^b
Phosphorus (mg/kg-soil)	92.4	93.6	14.4
Potassium (mg/kg-soil)	434.01	948.18	127.08
Calcium ($meq/100cm^3$ soil)	35.6 (77% of CEC)	29.2 (84% of CEC)	6.5 (64% of CEC)
Magnesium ($meq/100cm^3$ soil)	9.7 (21% of CEC)	3.5 (10% of CEC)	1.8 (18% of CEC)
Manganese (mg/kg-soil)	84.80	129.60	110.88
Zinc (mg/kg-soil)	5.08	3.44	6.12
Copper (mg/kg-soil)	2.78	5.26	1.06
Sulfur (mg/kg-soil)	189.00	1.36	1.48
Sodium ($meq/100cm^3$ soil)	12	0.5	0.0

[a] pH determined by North Carolina Department of Agriculture Agronomics Division.

[b] pH determined by pH-H_2O procedure [40].

Table 4.2. Comparison of Aerobic and Anaerobic ORP at 365-Days.

	Aerobic E_H (mV)	Anaerobic E_H (mV)
ED Samples:		
Water	400.0	64.7
Nitrate	446.2	381.6
Sulfate	455.1	84.7
Water + Formalin	208.8	103.7
Nitrate + Formalin	199.2	97.3
Sulfate + Molybdate	304.8	-17.0
SD Samples:		
Water	452.2	52.7
Nitrate	474.0	382.1
Sulfate	467.0	56.2
Water + Formalin	248.2	122.5
Nitrate + Formalin	233.6	113.4
Sulfate + Molybdate	324.6	74.4
SK Samples:		
Water	470.7	159.8
Nitrate	467.0	368.3
Sulfate	457.1	-5.8
Water + Formalin	319.4	151.5
Nitrate + Formalin	318.3	140.0
Sulfate + Molybdate	324.3	85.4

$E_H = E_{meas} + 222.3mV$, based on Ag/Ag-Cl reference electrode. All anaerobic E_H values are significantly different from aerobic ($p<0.01$). Note that nitrate is not an electroactive ion, therefore samples containing nitrate will not generate an expected effect on electrode measurement [77]. Formalin treatment also impacts E_H measurement, as addressed in Chapter 3.

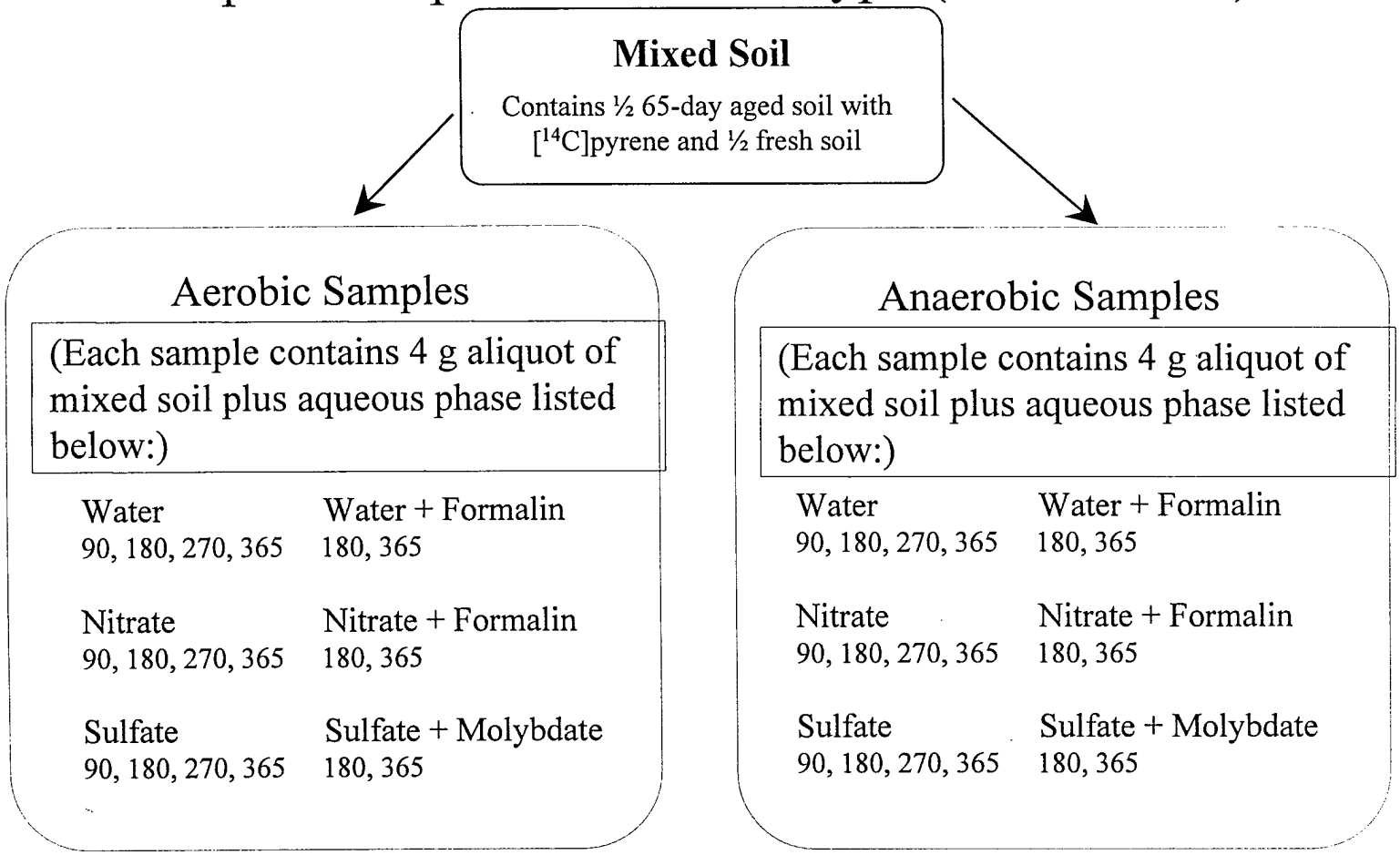

Figure 4.1. Sample set-up for each soil type. Each set of samples consisted of 3-replicates of each aqueous phase for every time-point (time in days listed under aqueous phase).

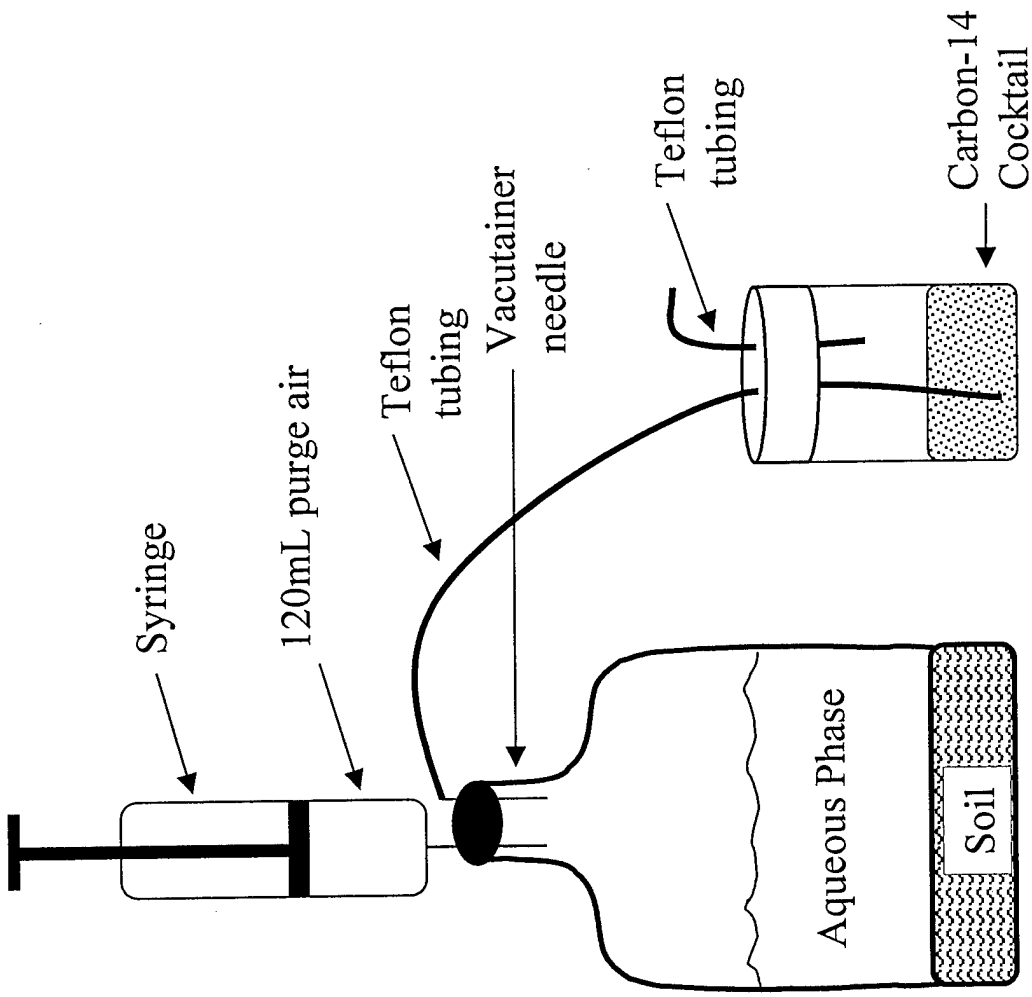

Figure 4.2. Diagram of headspace purge procedure used to quantify $^{14}CO_2$ in headspace of samples. Air was blown through the samples using a syringe, and the purged $^{14}CO_2$ trapped in carbon-14 cocktail.

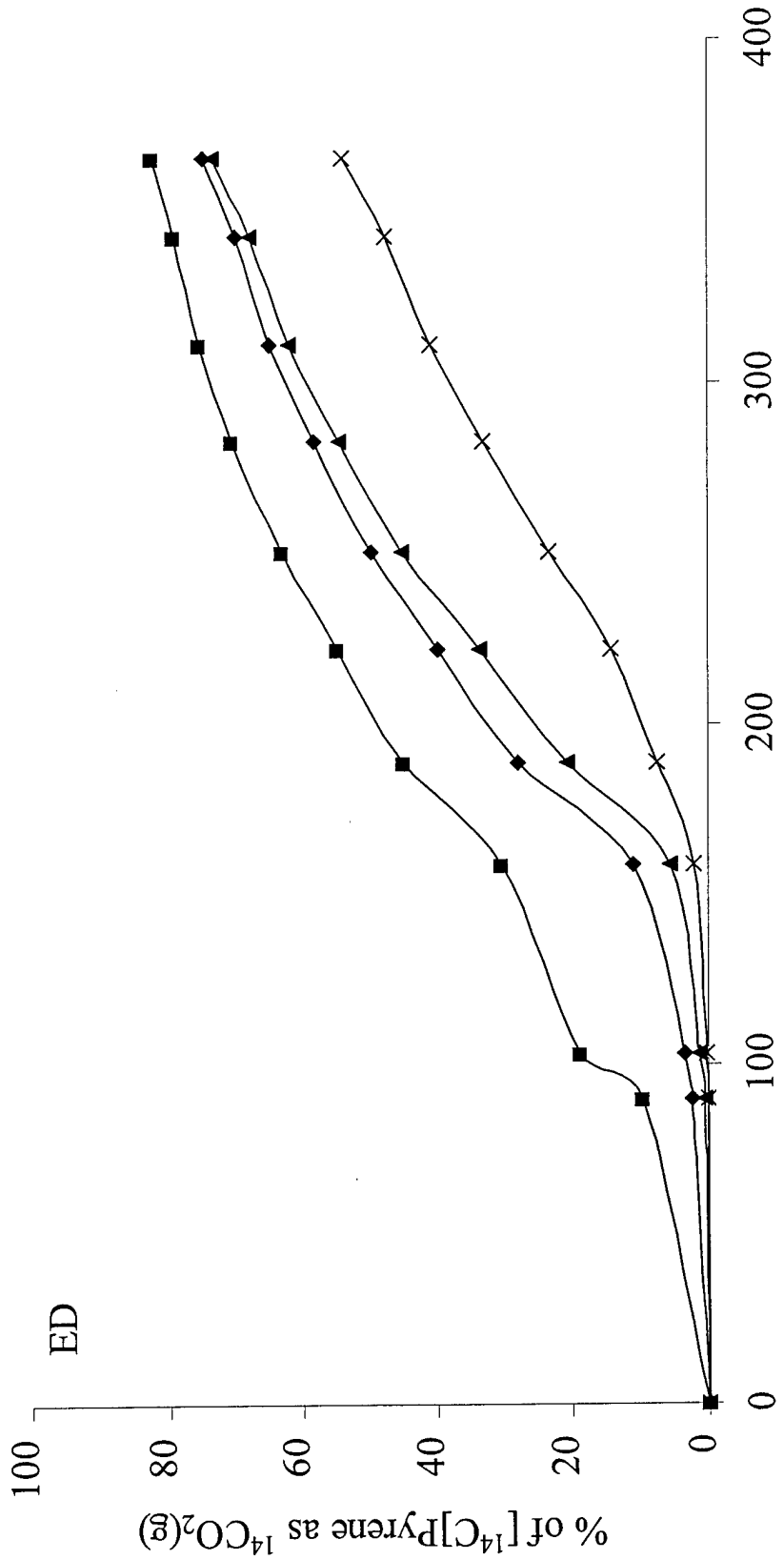

Figure 4.3. Cumulative percent $^{14}CO_2$ generated from the aerobic mineralization of [^{14}C]pyrene over time in ED soil. Symbols represent average of three samples: (◆) water, (■) NO_3^-, (▲) SO_4^{2-}, (×) SO_4^{2-} and molybdate.

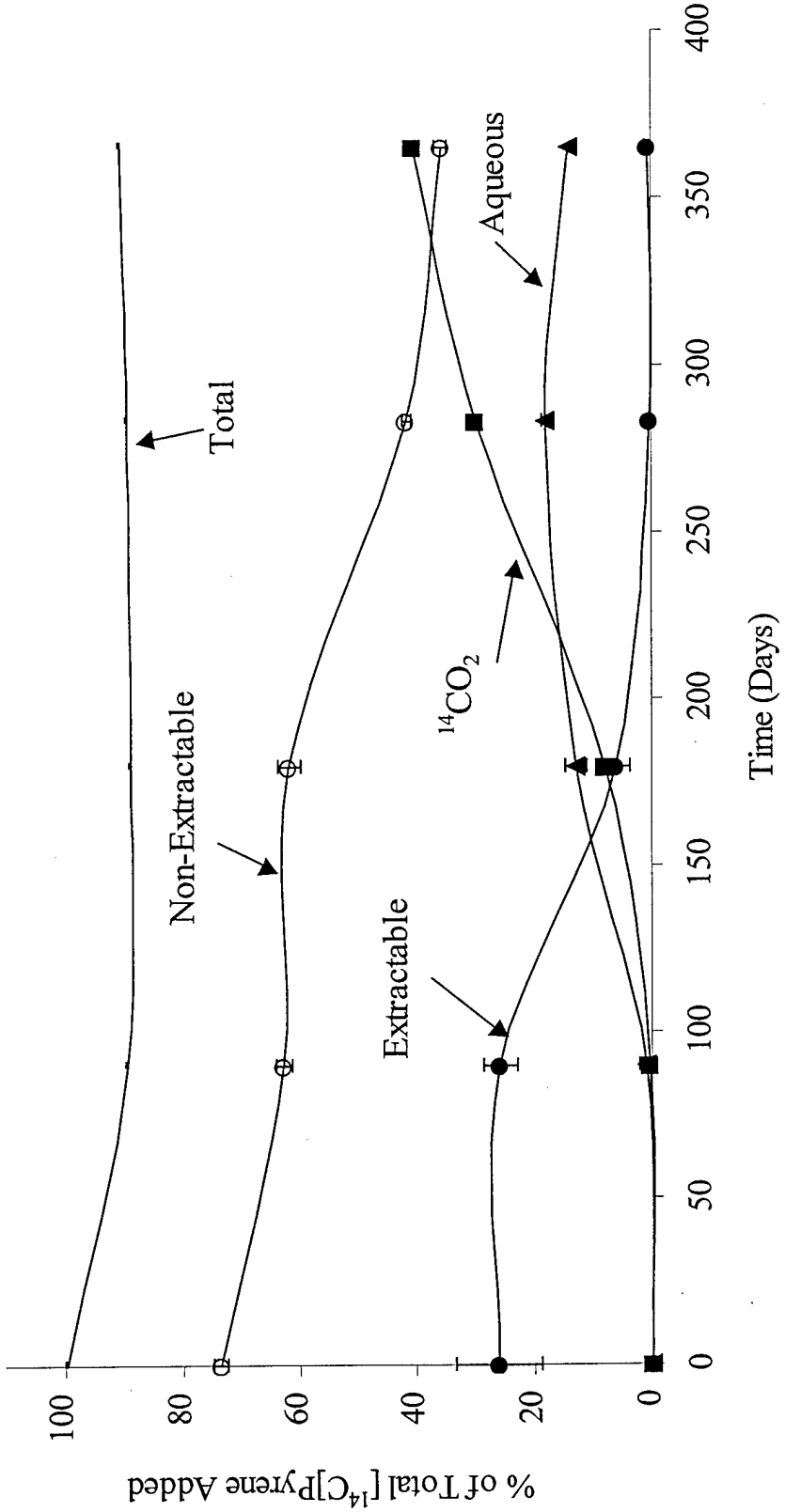

Figure 4.4. Percent of total [^{14}C]pyrene added for each parameter measured in SD soil incubated under aerobic conditions with water. Error bars represent one standard deviation from the mean of triplicate samples. Symbols represent average of three samples: (■) $^{14}CO_2$, (▲) aqueous phase, (●) extractable from soil, (○) non-extractable from soil, (-) total radiolabel recovered.

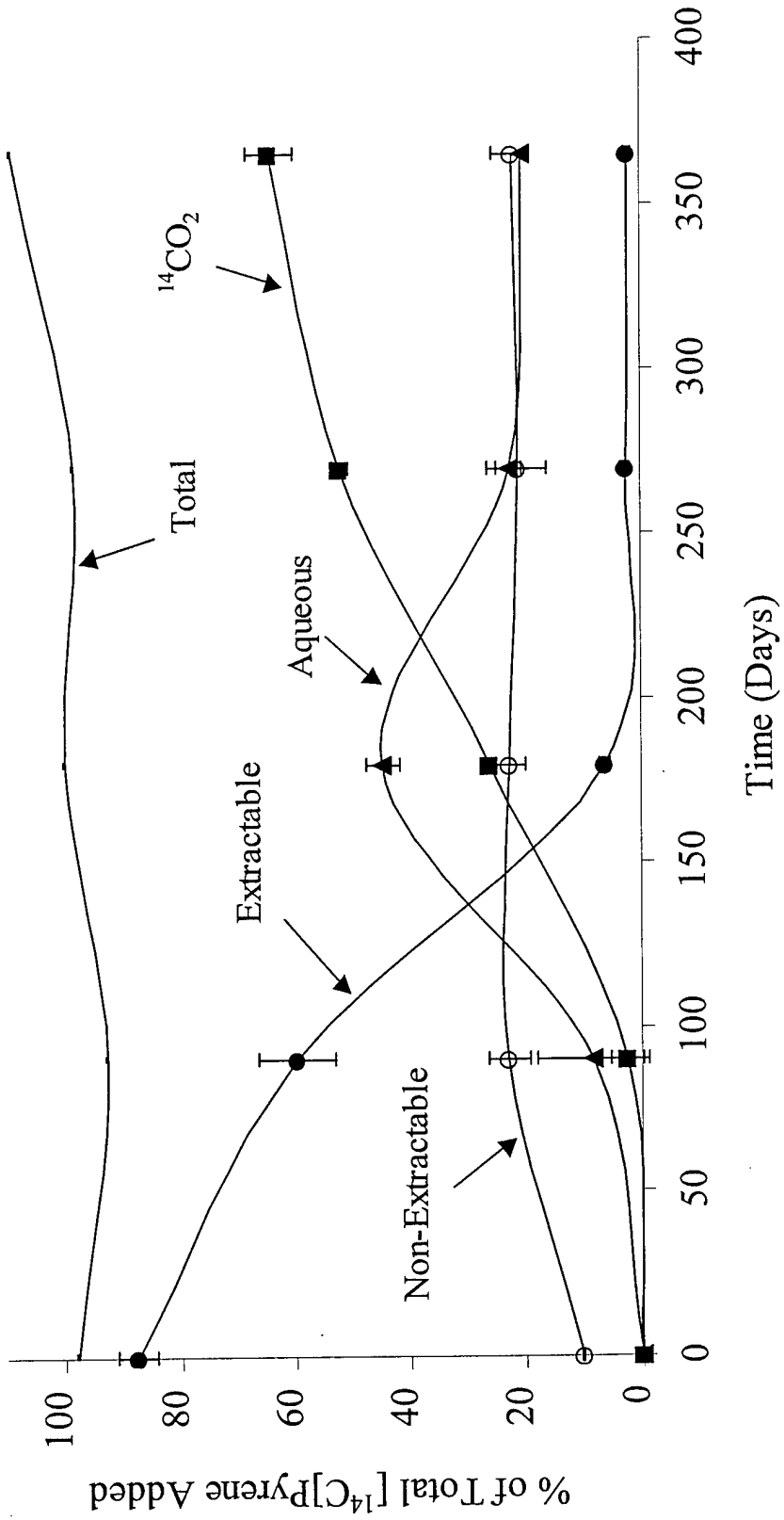

Figure 4.5. Percent of total [^{14}C]pyrene added for each parameter measured in ED soil incubated under aerobic conditions with water. Error bars represent one standard deviation from the mean of triplicate samples. Symbols represent average of three samples: (■) $^{14}CO_2$, (▲) aqueous phase, (●) extractable from soil, (○) non-extractable from soil, (-) total radiolabel recovered.

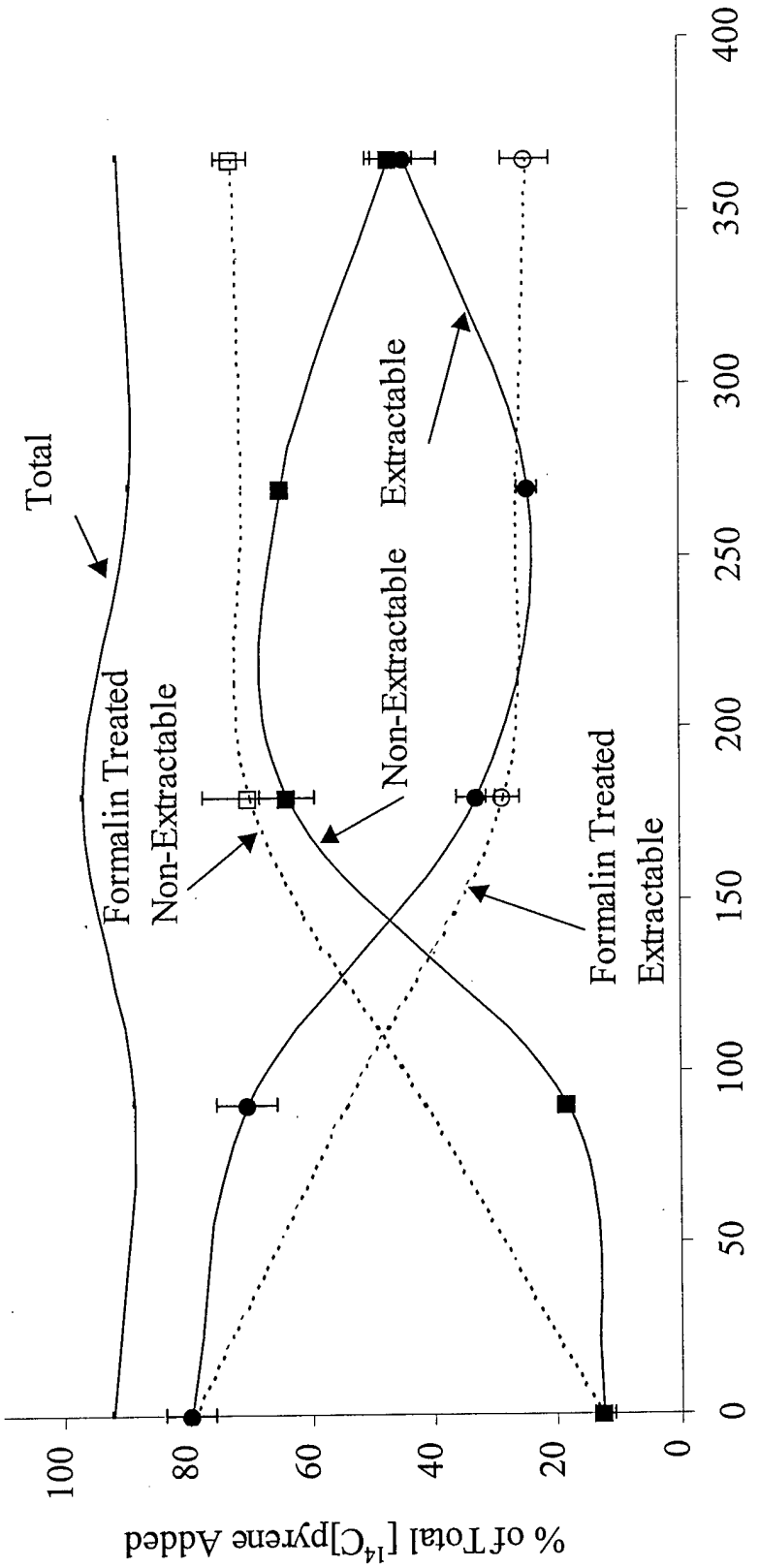

Figure 4.6. Percent of total [^{14}C]pyrene added extractable or non-extractable radiolabel from soil measured in SK soil incubated under anaerobic conditions with water (closed symbols) or 5% formalin (open symbols). Error bars represent one standard deviation from the mean of triplicate samples. Symbols represent average of three samples: (●) and (○) extractable from soil, (■) and (□) non-extractable from soil, and (-) total radiolabel recovered.

CHAPTER 5: SUMMARY AND CONCLUSIONS

5.1. SUMMARY OF FINDINGS

The proposed ORP related pH and microbial influences on organic material, and subsequent solubilization of PAH, were consistent with all experimental observations made in Chapters 3 and 4. The studies detailed in Chapter 3 demonstrated significant increases in aqueous phase PAH concentrations due to controlled anaerobic incubation. Methanogens were seen to have the greatest influence on aqueous PAH solubility, however, SRB may have a greater influence than methanogens when a suitable carbon source for methanogens is limited. PAH made more soluble under anaerobic conditions were available to be degraded or transformed under aerobic conditions.

The study detailed in Chapter 4 employing three distinctly different soils demonstrated that there were microorganisms in previously uncontaminated soils that were capable of degrading pyrene under aerobic conditions. For aged pyrene in SD soil, a one year incubation of pyrene in soil proceeded to a significant degree in terms of conversion of pyrene to CO_2, even when a high percent of the initial pyrene was firmly bound to soil in the non-extractable fraction. This study also showed that specific anaerobic conditions, especially those generated under nitrate and sulfate reducing states, significantly influenced the extractability of pyrene from some soils.

5.2. CONCLUSIONS

The persistence of PAH contamination has been investigated for many years. Most studies of high molecular weight PAH contamination have involved aerobic conditions. So, any research completed that uses anaerobic incubations provides valuable information concerning PAH behavior. The results presented in this dissertation emphasized the need to

consider the effects of anaerobic conditions when assessing the risk associated with a PAH contaminated site.

The first objective of this research was to examine the influence of controlled anaerobic conditions on the aqueous solubility of high molecular weight PAH during a one-year incubation of contaminated soil. Aqueous phase PAH concentrations increased with all methods used to generate anaerobic conditions, as compared to aerobic samples. The influences of anaerobic microorganisms were a significant finding in this research. The mechanisms of microbial attachment in soil have been extensively studied [70], but the microbial interaction with contaminated soil has not. The results presented in Chapter 3 are significant with respect to transport in the environment and bioavailability.

The second objective of this work was to assess aerobic bioavailability of PAH made more soluble under anaerobic conditions. The An/Air incubations presented in Chapter 3 clearly demonstrated that PAH made more soluble in the aqueous phase under anaerobic conditions were bioavailable under an aerobic environment. These results are important when considering a leachate plume, since highly reduced conditions are often associated with plumes, which may potentially increase transport in the environment. The results related to this objective demonstrated that if contaminants are transported to an aerobic location, degradation might occur.

The third objective of this research was to examine the influence of inorganic nutrient addition on pyrene bioavailability in three different soils under aerobic conditions. The extensive pyrene degradation observed in soils with no known history of PAH contamination was important since this indicates the ubiquitous nature of PAH-degrading microorganisms. The enhanced aerobic degradation rate in a desorption-limited SD soil by nitrate addition was also an important observation, since this finding may impact remediation strategies.

The final objective of this work was to examine the effect of controlled anaerobic conditions and electron acceptor additions on pyrene bioavailability in three different soils. The results of this study also highlighted the necessity to consider the metabolic community when assessing bioavailability of PAH in soil, especially under nitrate-reducing and sulfate-reducing conditions.

These studies emphasize the importance of considering the microbial community in a system since they will influence PAH directly or indirectly through pH and DOM alteration. Because strongly reduced conditions are usually established in the leachate plume associated with hazardous waste sites [52, 66], the generation of an anaerobic environment must be considered. As the results of this research have shown, a decreased redox state alters the soil structure in such a manner as to make the PAH more aqueous phase soluble. For example, if remediation strategies involving the application of topsoil are used, as in the case of the Reilly Tar and Chemical Corporation Superfund Site, the site may be anaerobic more of the time, resulting in a more mobile source of PAH contamination. Significant transport of PAH contamination from this site may occur. These anaerobic influences will have important implications on bioavailability, especially if organic matter associated-PAH migrate to a location with sufficient oxygen and nutrient requirements for biodegradation. An understanding of conditions that may influence the fate and sorption of organic contaminants is necessary to delineate the hazards posed to the public and the environment [13].

CHAPTER 6: FUTURE RESEARCH NEEDS AND LESSONS LEARNED

6.1. FUTURE RESEARCH

The results of these experiments demonstrate the need to observe the effects of anaerobic incubations in greater detail. Some specific thoughts are listed here:

- Microorganisms responsible for generating pH and ORP changes should be identified. The nature of the responsible microbe will allow for a more accurate risk assessment when observing contaminated sites.
- Anaerobic microorganisms alter the structure of the soil releasing aqueous soluble PAH. These mechanisms should be studied to determine how anaerobes exert their influence on soil and soil contamination.
- A radiolabel tracer study should be completed to verify the bioavailability suggested in the An/Air experiments.
- Further investigations should be completed to determine if these same ORP and pH changes occur predictably in other soils.
- Further studies should be completed with other soils under various headspace and electron acceptor conditions. Since anaerobic conditions generated in the study employing three different soils used N_2:CO_2(g) headspace gas, any pH alteration was masked by the carbonate buffering system.

6.2. LESSONS LEARNED

For anyone just starting out in the world of anaerobic research, there are some pieces of information I learned while completing this study:

- Always measure headspace gas composition when dealing with anaerobes since metabolic products can assist in the microbial identification.

- Carefully consider the components of your medium. As I found, you can unintentionally select for a consortia of organisms (which turned out to be a beneficial error in this research).
- The glove bag is an excellent piece of equipment that facilitates the assembly of anaerobic microcosms. However, use care in setting up microcosms in the glove bag since there will be up to 4% $H_2(g)$ in the headspace, which can stimulate metabolic activity in some microorganisms.
- There is no perfect method to inhibit or "kill" microorganisms without significantly altering the soil or aqueous phase of microcosms. Carefully examine results to distinguish between side-effects due to the metabolic inhibitor and effects due to a lack of microbial activity.

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